Characterisation of TRIM5α and Related Retroviral Restriction Factors

PhD thesis

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Torsten Schaller

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I, Torsten Schaller confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

London, 02.06.2009
Torsten Schaller

„Mehr als die Vergangenheit interessiert mich Zukunft, denn in ihr gedenke ich zu leben.“
Albert Einstein

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Abstract

Retroviruses possess an RNA genome that is reverse transcribed into DNA and becomes integrated into the host genome during infection. Human immunodeficiency virus 1 (HIV-1) belongs to the genus lentivirus of the retroviridae family and is a major human pathogen with more than 50 million infected people worldwide to date. Lentiviruses have very narrow host ranges. Cross-species lentiviral transmissions are rare, due to the ability of mammals to counteract viral infections by the innate immune system, which includes intracellular factors that block virus replication. Of these, the murine protein Fv1 and the tripartite (RBCC) motif protein family member TRIM5α potently inhibit retroviral infections early after virus cell entry.

This study presents evidence that antiviral activity exerted by TRIM5α is conserved across mammals. Rabbit cells are strongly non-permissive to infections by certain retroviruses including HIV-1. The study identifies a TRIM5α orthologue in rabbits, which possesses antiretroviral activity. Phylogenetic analysis demonstrates that rabbit TRIM5 is a true orthologue and clusters with other mammalian TRIM5 genes.

Remarkably, TRIM5 fusion proteins with the prolyl-peptidyl isomerase cyclophilin A (CypA) have independently evolved twice by retrotransposition of a CypA cDNA into the TRIM5 genomic locus, generating a viral restriction factor with a CypA binding domain. This study shows that CypA fusion proteins with RBCC domains of other TRIM proteins also function as restriction factors against HIV-1. Furthermore, fusion of CypA to Fv1 enables the restriction of HIV-1 and FIV. Insights into the mechanism of TRIM5α restriction are also provided in this study. Functional analyses of TRIM5α and TRIMCyp deletion mutants, as well as fusion proteins of Fv1Cyp with the TRIM5α RING and B-box domains, indicate that the RING and B-box domains mediate the proteasome dependent block to reverse transcription during TRIM5α/TRIMCyp restriction. The results suggest that the major antiviral activity exerted by TRIM5α is a RING dependent block to reverse transcription, whereas the major activity of TRIMCyp is independent of RING function.

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Abbreviations

agm	African green monkey
AIDS	Acquired immunodeficiency syndrome
APOBEC3	Apolipoprotein B mRNA-editing catalytic polypeptide 1-like protein 3
Bst-2	Bone marrow stromal protein 2
CA	Capsid protein
cDNA	Complementary DNA
cpz	Chimpanzee
CRFK	Crandell Reese feline kidney
CsA	Cyclosporine A
Cyp	Cyclophilin
DNA	Deoxyribonucleic acid
EIAV	Equine infectious anaemia virus
Env	Envelope
FACS	Fluorescence activated cell sorting
FIV	Feline immunodeficiency virus
Fv1	Friend virus susceptibility 1
Gag	Group specific antigens
GFP	Green fluorescent protein
gor	Gorilla
Gp	Glycoprotein
gsn	Greater spot-nosed
h	Hours
HIV	Human immunodeficiency virus
HRP	Horse reddish peroxidase
Hu	Human
IFN	Interferon
IN	Integrase
IU	Infectious units
kb	Kilobase
kDa	Kilodalton
LTR	Long terminal repeat
MA	Matrix
Mac	Macaque
min	Minutes
ml	Millilitre

MLV	Murine leukaemia virus
NC	Nucleocapsid
Nef	Negative factor
NES	Nuclear export signal
NLS	Nuclear localisation signal
NPC	Nuclear pore complex
nt	Nucleotide
Om	Owl monkey
OPCR	Overlap PCR
PBL	Peripheral blood lymphocytes
PCR	Polymerase chain reaction
PIC	Pre-integration complex
Pol	Polymerase
RACE	Rapid amplification of cDNA ends
Rb	Rabbit
RELIK	Rabbit endogenous lentivirus type K
Rh	Rhesus
RING	Really interesting new gene
RT	Reverse transcription
RTC	Reverse transcription complex
s	Seconds
Sab	Sabaeus
SDM	Site directed mutagenesis
ShRNA	Short hairpin ribonucleic acid
SIRC	Statens serum institute rabbit corneal
SIV	Simian immunodeficiency virus
Sm	Squirrel monkey
snp	Single nucleotide polymorphism
stm	Stump-tailed macaques
tan	Tantalus
TCyp	TRIMCyp
TRIM	Tripartite motif
tRNA	Transfer RNA
Vif	Viral infectivity factor
Vpu	Viral protein U
VSV-G	Vesicular stomatitis virus G protein

1. Introduction

1.1. HIV-1 is the prototypic lentivirus

Human immunodeficiency virus 1 (HIV-1), the etiologic cause of the acquired immunodeficiency syndrome (AIDS), was first isolated in 1983 from the lymph node of a person with lymphadenopathy by Francoise Barré-Sinoussi, Jean-Claude Chermann and Luc Montagnier at the Institute Pasteur in Paris [1]. However, the identified isolate, called Bru, replicated poorly in cell culture and was, unrecognised by researchers at the Institute Pasteur, contaminated by a more robustly and quickly replicating second AIDS isolate, called Lai. Samples labelled as Bru but contaminated with Lai were sent to other labs including the lab of Robert Gallo at the U.S. National Institute of Health. Almost one year after the original discovery of Bru, the Gallo lab reported the isolation of a T-cell tropic retrovirus robustly replicating in cell culture, named human T-cell lymphotropic virus type III (HTLV-III), which subsequently was identified to be similar to the isolate Lai [2]. The lab around Simon Wain-Hobson reconstructed the chain of events [3] and Barré-Sinoussi and Montagnier obtained the Nobel Prize for Medicine for their discovery in 2008. Early names for the discovered virus were LAV, lymphadenopathy-associated virus (Institute Pasteur Paris); HTLV-III (U.S. NIH) and ARV, AIDS-associated retrovirus (University of California, San Francisco), but in 1986 the name human immunodeficiency virus was assigned due to the virus's relatedness with a group of known lentiviruses. Three years after the discovery of HIV, a second distantly related human immunodeficiency virus (HIV-2) was discovered in a distinct region in West Africa, which also caused AIDS in humans, however in fewer numbers of infected people compared to HIV-1 [4].

One of the oldest known lentiviruses is equine infectious anaemia virus, EIAV, which causes an autoimmune haemolytic anaemia in horses (Table 1) and was found in 1904 as a filterable agent causing anaemia in horses [5] although its relatedness to HIV-1 was only discovered in 1986 [6]. After the discovery of HIV, scientists identified a similar lentivirus in cats (FIV, feline immunodeficiency virus formerly known as feline T-lymphotropic virus) [7] and in diverse non-human primates (SIV, simian immunodeficiency virus) [8-11].

Due to its impact on human health, HIV-1 is the most exhaustively studied lentivirus to date but still is only partially understood.

1.2. The origin of HIV-1

HIV-1 originates from a simian immunodeficiency virus infecting chimpanzees (*Pan troglodytes troglodytes*) designated SIVcpz*Ptt,* which is thought to have crossed species three times resulting in the emergence of HIV-1 virus group M, which caused the AIDS pandemic and HIV-1 group N, which is geographically restricted to a region of Cameroon (FIG 1) [12, 13]. In addition, recent data suggests that SIVcpz*Ptt* may also be the origin of HIV-1 group O (FIG 1) [14]. SIVcpz*Ptt* is also thought to have crossed species infecting western low land gorillas (*Gorilla gorilla gorilla*), leading to the emergence of SIVgor [14, 15]. In contrast, HIV-2 is most closely related to SIVsm (FIG 1), indicating that HIV-2 infection in humans was a result of a zoonotic transmission of SIVsm from sooty mangabeys to humans [16]. Interestingly, the earliest lymph node biopsies, both derived from Léopoldville (today Kinshasa, Democratic Republic of the Congo), in which HIV-1 group M virus could be detected, date from 1959 and 1960 [17, 18]. The divergence of the two isolated sequences (12 % sequence difference) points to a much earlier date of zoonotic transmission of SIVcpz*Ptt* from chimpanzees to human, suggesting that HIV-1 may have been circulating long before 1960.

Lentiviruses have narrow host ranges and HIV-1 only replicates in humans, chimpanzees [19] and possibly gorillas [15]. Interestingly, early studies suggested that HIV-1 infection of chimpanzees almost always appears non-symptomatic and only one HIV-1 infected chimpanzee from the Yerkes cohort showed progression of HIV-1 infection to AIDS [20, 21]. However, recent reports increasingly suggest that infection of chimpanzee with SIVcpz*Ptt*, the closest relative of HIV-1, leads to a symptomatic infection and can progress to AIDS [22]. SIVcpz*Ptt* can be transmitted horizontally between chimpanzees and infections increase the risk for mortality by 15-fold [22]. Moreover, significantly more chimpanzee babies born to SIVcpz*Ptt* infected mothers die compared to infants born to uninfected mothers, suggesting mother to child transmission of SIVcpz*Ptt* [22].

Although this may provide a new approach for an animal model, chimpanzees as well as gorillas are endangered, experiments are cumbersome and expensive and therefore these animals do not present a suitable model for HIV-1/AIDS research. Many attempts have been undertaken to find a small animal model. Gibbons (*Hylobates lar*) and pigtailed macaques (*Macacca nemestrina*) have been infected with HIV-1 and studied as possible animal models for infection [23, 24]. However, the replication of HIV-1 remained poor, and in all cases infections were non-pathogenic and did not lead to AIDS. In the 1970's

before HIV was discovered, experiments studying the disease Kuru led to the transmission of an SIV from sooty mangabeys (sm) to rhesus macaques (mac) and the emergence of two viruses (SIVstm and SIVmac) that caused AIDS in sooty mangabeys as well as in rhesus macaques (Table 1) [25]. In addition, SIVsm and SIVmac infection of cynomolgus macaques causes AIDS, and these systems are studied as surrogate models for human AIDS [26-28].

Furthermore, SIV-HIV-1 chimaeric virus infection of macaques has been studied as a model for HIV-1 infection. This system was used for attempts to develop an attenuated live vaccine, and has been proven useful to test reverse transcriptase inhibitors *in vivo* [29, 30]. Early studies of infection of pigtailed macaques (*Macacca nemestrina*) with HIV-1 showed that they were partially permissive for HIV-1 replication as well as replication of HIV(NL-DT5R), a chimaeric mutant in which 7 amino acids of CA and the entire *vif* gene were replaced by SIV sequences [23, 31-33]. More recently, pigtailed macaques were infected with a SIV-HIV-1 chimaera in which only the HIV-1 *vif* gene was replaced by SIVmac or HIV-2 *vif* [34]. The study showed that the chimaeric virus (stHIV-1) was able to cause acute viremia in infected animals similar to viremia levels seen in HIV-1 infected humans and that virus replication was persistent over several months but was eventually controlled at least partially by CD8+ T-cell activity [34]. In contrast to most Old World monkeys, which express a TRIM5α that blocks HIV-1 infection, pigtailed macaques express a TRIM5-cyclophilin A fusion protein that is unable to restrict HIV-1 infectivity and may counteract endogenous TRIM5α [35]. Furthermore, a study infecting cynomolgus and rhesus macaques with SIV-HIV-2 Env or Gag/Pol chimaeric viruses demonstrated that virus could be recovered from rhesus macaques up to twelve weeks after challenge, indicating persistence of the chimaeras in these animals [36].

In addition to primate animal models, FIV infection of cats has been proposed as a surrogate model for HIV-1 infection in humans [37-41]. Cats are suitable since after infection with FIV they develop AIDS like symptoms including a self-limiting acute disease, followed by an asymptomatic phase with loss of CD4 and CD8 T-cells and pre-AIDS disorders (Table 1) [37, 42]. Therefore they are currently used to test different vaccine strategies against FIV, which may reveal novel ways for HIV-1 vaccine development.

Virus		Host Species	Disease
HIV-1	Human immunodeficiency virus 1	Human	AIDS
HIV-2	Human immunodeficiency virus 2	Human	AIDS
SIVmac	Simian immunodeficiency virus	Macaques	AIDS
SIVcpz*Ptt*	Simian immunodeficiency virus	Chimpanzees	(AIDS?)
SIVgor	Simian immunodeficiency virus	Gorillas	*unknown*
SIVsm	Simian immunodeficiency virus	Sooty mangabeys	*unknown*
SIVrcm	Simian immunodeficiency virus	Red capped mangabeys	*unknown*
SIVagmTan	Simian immunodeficiency virus	Tantalus monkeys	*unknown*
SIVagmSab	Simian immunodeficiency virus	Vervet monkeys	*unknown*
FIV	Feline immunodeficiency virus	Cats	AIDS
EIAV	Equine infectious anaemia virus	Horses	Anaemia
MLV	Murine leukaemia virus	Mice	Cancer

Table 1 Retroviruses, host species and disease caused in the host

Depicted are retroviruses, the hosts in which these viruses are found and the diseases the viruses cause in its hosts. Gag expression vectors from grey highlighted viruses have been analysed in this study.

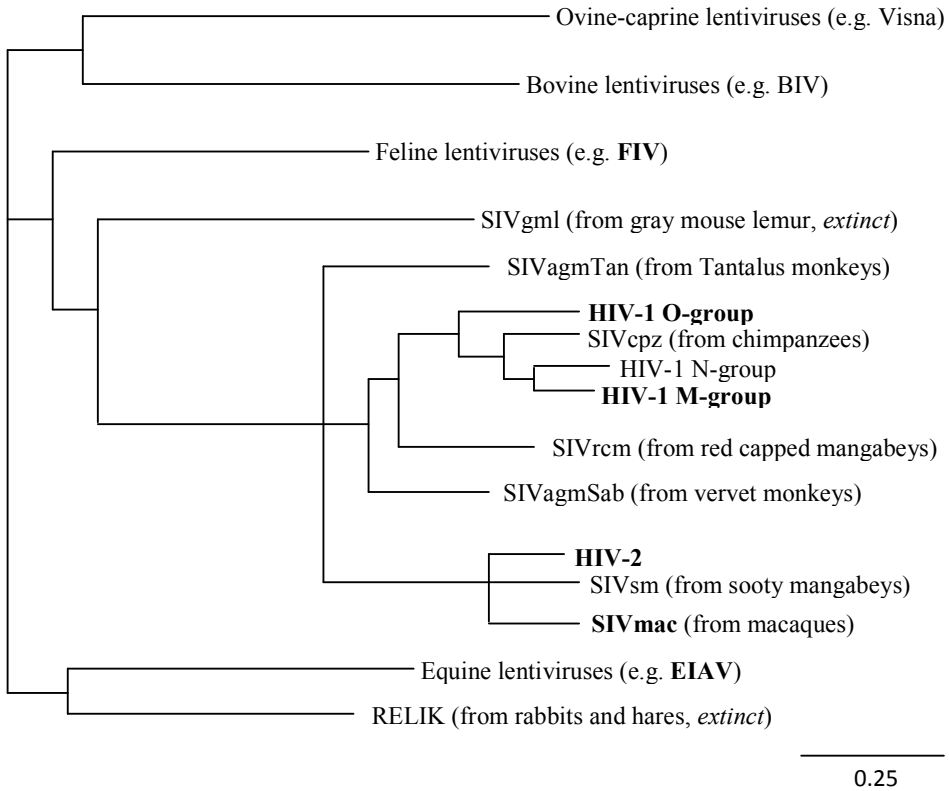


FIG 1 Phylogenetic tree of lentiviruses

Depicted is the phylogenetic relationship between lentiviruses. Gag expression vectors from bold viruses were analysed in this study. RELIK, rabbit endogenous lentivirus; EIAV, equine infectious anaemia virus; BIV, bovine immunodeficiency virus; FIV, feline immunodeficiency virus; SIV, simian immunodeficiency virus; HIV, human immunodeficiency virus; agm, African green monkey. Scale indicates substitutions per site. Figure adapted from [43].

1.3. Genomic organisation of HIV-1

The HIV-1 genome is diploid consisting of two RNA molecules of positive polarity each of approximately 9 kb in length. Like cellular mRNAs both viral RNA molecules are 5' capped with 7-methylguanosine and possess a 3' polyA tail [44]. The genomic RNAs are packaged into nascent HIV-1 particles and are the template for reverse transcription in an infected cell, generating viral DNA that is integrated into the host genome establishing a provirus. The synthesis of the two full-length genomic RNAs is mediated from the provirus via a promoter region in the 5' long terminal repeat (LTR) region (FIG 2). Viral genomic RNAs contain *cis*-acting elements like the trans-activating responsive region (TAR), the Rev responsive element (RRE), the primer binding site (PBS), the packaging signal Ψ and the 3' and central polypurine tracts (PPT), that are essential for diverse steps in the virus life cycle. HIV-1 encodes the typical retrovirus polypeptides Gag (group-specific antigen), Pol (polymerase) and Env (envelope) as well as additional proteins Vif, Vpr, Vpu, Nef, Tat and Rev. All the proteins are expressed from different splice forms of a single RNA transcript and are important for a natural occurring infection.

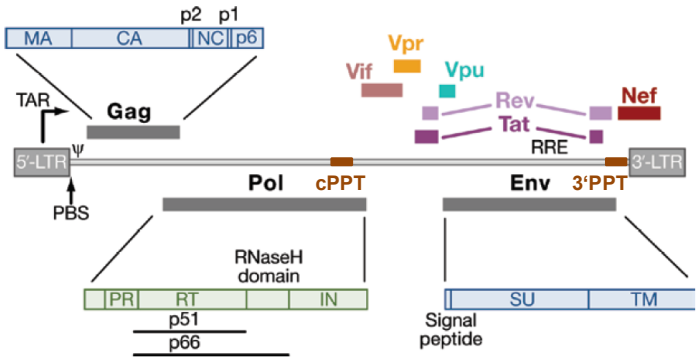


FIG 2 Genetic organisation of HIV-1

HIV-1 consists of the polyproteins Gag, Pol and Env and has additional auxiliary proteins Vif (viral infectivity factor), Vpr (viral protein R), Vpu (viral protein unique), Rev (regulator of virion expression), Tat (transactivator of transcription) and Nef (negative factor). The 55kDa Gag polyprotein consists of the proteins MA (matrix), CA (capsid), the spacer protein p2, NC (nucleocapsid), spacer peptide p1 and the p6 protein. Cleavage of Gag into the individual proteins occurs after virus assembly in a process referred to as maturation. Pol is synthesised as a 160kDa Gag-Pol protein and encodes the viral protease (PR), the reverse transcriptase (RT), which is expressed as two different subunits p51 and p66 building a functional dimer, and the integrase (IN). The Env protein has an N-terminal signal peptide sequence, and is cleaved into gp120, the surface protein (SU), and gp41, the transmembrane protein (TM), both proteins being involved in attachment to cellular receptor/ co-receptor complexes and membrane fusion of viral and cellular membrane. Viral *cis* acting elements include the two long terminal repeats (LTR's), the primer binding site (PBS), the TAR stem loop that is bound by Tat for transcription activation, the Rev responsive element (RRE) which is bound by Rev mediating nuclear export of partially spliced and unspliced RNAs, a 3' and a central polypurine tract (PPT) and the RNA packaging signal Ψ. Figure adapted from [45].

1.4. The HIV-1 life cycle

The HIV-1 life cycle comprises several steps including the attachment of the virus to the cell, the release of its capsid into the cytoplasm, reverse transcription of the viral RNA into DNA, trafficking and nuclear import of pre-integration complexes, integration of the DNA into the host genome establishing the provirus, transcription and nuclear export of mRNAs as well as genomic RNAs, synthesis of viral proteins and virus assembly at the plasma membrane, and a final step of maturation to generate infectious progeny virions (FIG 3).

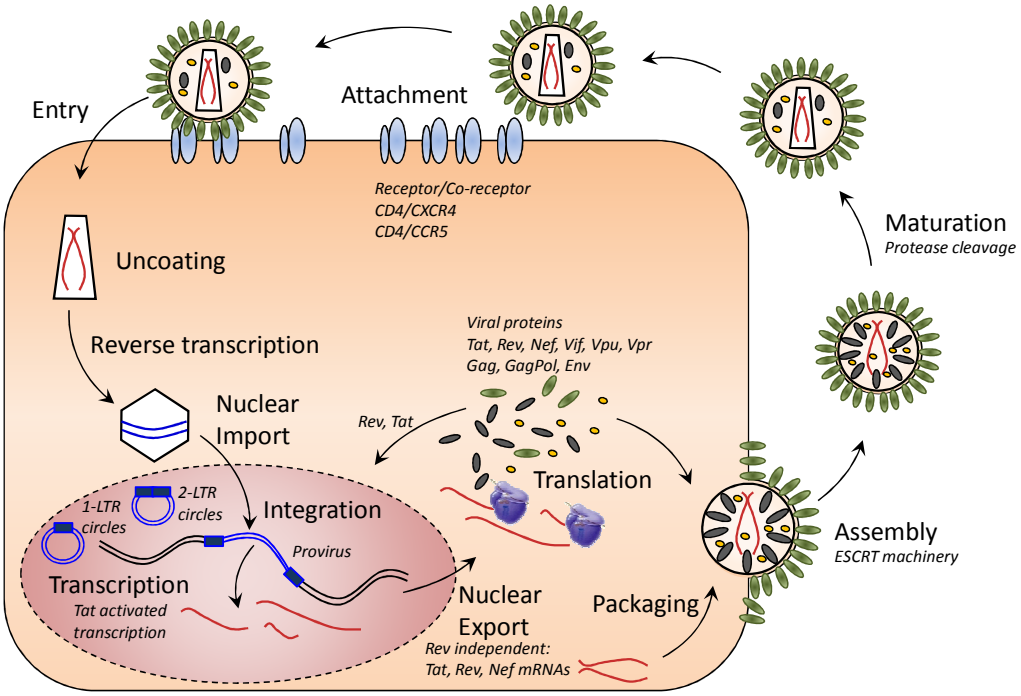


FIG 3 Life cycle of HIV-1

HIV-1 entry is initiated by the attachment of the gp120 envelope protein to the CD4 receptor and subsequent interaction with the co-receptors of the family of chemokine receptors. Fusion of host and virus membrane occurs with help of the fusion peptide in gp41. After membrane fusion the capsid is released into the cytoplasm where it undergoes a poorly understood process referred to as uncoating before the viral genome consisting of two single stranded RNAs of positive polarity is copied into one strand of double stranded DNA by the enzyme reverse transcriptase. The viral DNA is transported into the nucleus where it is integrated into the host genome by the viral enzyme integrase. In addition, two forms of circularised viral DNA are found, 1-LTR and 2-LTR circles. Proviral transcripts encoding for Rev and Tat are spliced, mRNAs are exported and synthesised Rev and Tat proteins are transported into the nucleus where they ensure efficient viral transcription (Tat) and the export of partially spliced mRNAs and genomic RNAs (Rev). Exported viral mRNAs are translated into Gag, GagPol and Env proteins and full-length viral genome RNA is packaged into virions. Assembly and budding of new virus is mediated by the host ESCRT-I/II/III machineries at the plasma membrane. After virus release, HIV-1 undergoes a maturation process during which Gag is cleaved and the cone shaped capsid shell is formed.

1.4.1. Attachment of HIV-1 and cell entry

The first step in the life cycle of HIV-1 is its attachment to the cell (FIG 3). HIV-1 infects CD4+ T-cells as well as macrophages using CD4 as a receptor [46]. CD4 is an integral membrane protein of the Ig superfamily and contains four Ig-like domains. CD4 associates with the T-cell receptor (TCR) and participates in signal transduction after recognition of MHC class II bound antigens by the TCR. The involvement of CD4 in the life cycle of HIV-1 was first observed when CD4+ Jurkat T-cells formed giant multinucleated syncytia in response to the glycoproteins of HIV-1 [46, 47]. The existence of at least one co-receptor for HIV-1 was considered by the observation that mouse cells did not become susceptible for HIV-1 after expression of human CD4. The co-receptors CXCR-4 and CCR5 were only discovered in 1996 [48, 49]. Depending on the co-receptor usage one can distinguish between CCR5 tropic and CXCR-4 tropic viruses as well as viruses that have a dual tropism. During the early stage of HIV-1 infection predominantly virus using CCR5 is found, whereas CXCR-4 tropic viruses occasionally arise at a later stage during the progression towards AIDS [50]. CXCR-4 and CCR5 are integral membrane proteins and belong to the class of chemokine receptors that bind the chemokines SDF-1 (CXCR-4) and RANTES, MIP-1α and MIP-1β (CCR5), respectively [51]. In addition, the chemokine receptor CCR3 has been suggested as another co-receptor for HIV-1 [52].

Interestingly, some people are partially resistant to infection by HIV-1 due to a homozygous 32 bp deletion in the coding region of their CCR5 genes leading to a truncated, non-functional receptor [53, 54]. This mutated CCR5 allele not only prevents infection in people that are homozygous for the deletion. People that are heterozygous for CCR5d32 seem to have a slower CD4+ T-cell decline and progress more slowly to AIDS [55-57]. The identification of the co-receptors for HIV-1 entry has led to the development of antagonising drugs, e.g. Maraviroc for CCR5, which has recently passed clinical trial phase III [58]. However, mutants resistant to Maraviroc have been reported and were mapped to the variable loop 3 (V3) of gp120 as well as to the fusion peptide of gp41 [59]. CXCR-4 antagonists have also recently been developed and new drugs are under clinical evaluation [60, 61]. However, CXCR-4 antagonists like AMD3100 may be less suitable than CCR5 antagonists since several side effects on normal cell functions have been observed [62].

HIV-1 binding to the cellular receptor/ co-receptor complex is mediated through its envelope glycoproteins gp120 and gp41, which are derived from a gp160 precursor protein that is cleaved in virus producing cells by the cellular peptidase furin and other proteases [63]. Gp160 is synthesised in the rough endoplasmic reticulum where it gets heavily glycosylated at asparagine residues. The protein then undergoes modification in the Golgi in which the mannose glycosylation is further processed and the surface (SU/ gp120) and transmembrane (TM/ gp41) proteins are generated by endoproteolytic cleavage, an event that is essential for the generation of infectious virions [64]. Both proteins remain non-covalently attached and are transported to the plasma membrane where they form trimers, also referred to as spikes. Each newly formed virion incorporates approximately 8-10 trimers of gp120/gp41 [65]. Mutations that affect the cleavage of the gp160 precursor or that affect the association of the gp120 and gp41 subunits render HIV-1 non-infectious [66]. Gp120 and gp41 have distinct functions in virus attachment to the cell and fusion of the virus membrane with the host cell membrane. As an initial step gp120 binds to CD4 (FIG 4A). After gp120 binding to CD4, a binding site in gp120 is exposed, which attaches to the co-receptor (FIG 4B). Binding to the co-receptor leads to a conformational change in gp41 so that the formerly unexposed fusion peptide becomes exposed. This fusion peptide inserts into the target cell membrane and mediates membrane fusion. In a process similar to structural changes seen for SNARE proteins involved in membrane fusion of vesicles, the two alpha helices (HR1 and HR2) of a gp41 monomer form a rod-like hairpin structure thereby bringing viral and cellular membranes closer together so that fusion can occur (FIG 4C). The resulting post-fusion structure of gp41, a six-helix bundle, is depicted in FIG 5. The events occurring at this stage are also strikingly similar to events happening during influenza HA mediated membrane fusion however, unlike for HA they are not dependent on a low pH. Recent data however suggests that the fusion of viral and host cell membranes occurs in endosomes and not directly at the plasma membrane [67]. Fusion of the viral and cellular membrane leads to the release of the virus capsid shell into the cytoplasm.

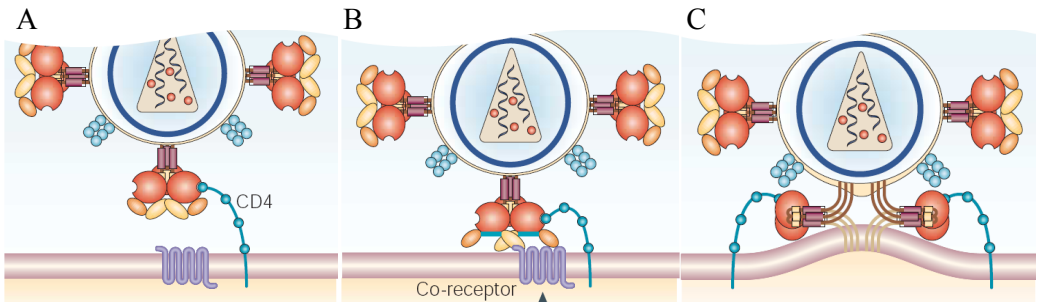


FIG 4 Attachment of HIV-1 and events occurring during membrane fusion

A) HIV-1 attaches to cell membrane associated CD4 via the gp120 protein. B) Attachment brings the virus closer to the cell membrane and induces a conformational change in the gp120 protein so that it can bind to the co-receptor C) Binding to the co-receptor leads to the exposure of the gp41 fusion peptide which inserts into the host cell membrane. Formation of a helix bundle leads to annealing of virus and cell membranes causing membrane fusion. Fusion is thought to occur after endocytosis. Figure adapted from [68].

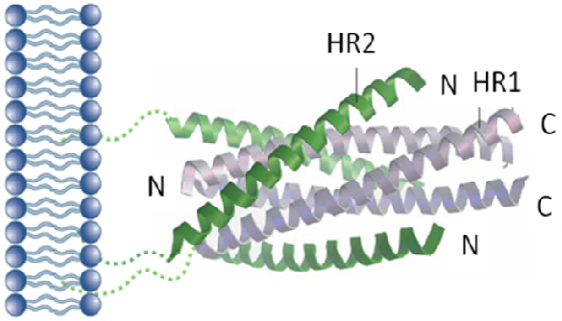


FIG 5 Six-helix bundle gp41 post fusion structure

HIV-1 glycoprotein 41 (gp41) contains an N-terminal fusion peptide that is inserted into the target cell plasma membrane. Each monomer of gp41 has two helical domains (HR1 and HR2), which form helical bundles during membrane fusion. In the fused membrane, gp41 is found as a trimeric structure in a six-helix bundle. Figure adapted from [69].

1.4.2. Reverse transcription of HIV-1

During the process of HIV-1 membrane fusion with the host cell the viral core is released into the cytoplasm and a poorly characterised process described as uncoating takes place (FIG 3). It is believed that HIV-1 cores disassemble in the cytoplasm since HIV-1 CA is not found in nuclear fractions of infected cells [70]. However, some evidences suggest that uncoating occurs in proximity to nuclear pore complexes [71]. In addition, recent work suggest that HIV-1 CA is involved in nuclear import of HIV-1 pre-integration complexes, suggesting that fractions of CA might enter the nucleus [72].

It is at the early step after infection that cellular host proteins like TRIM5α, Cyclophilin A and probably other yet unidentified factors act to enhance or inhibit the virus. However, the molecular details of the mechanisms of these factors remain unclear.

For successful infection retroviruses need to transcribe their genomic RNA into a double stranded DNA molecule, which is then integrated into the host genome. All retroviruses undergo the process of reverse transcription shortly after they have entered the target cell and this process is normally completed within 8 to 12 h after infection [73]. Reverse transcription occurs in a complex of viral RNA and proteins, referred to as the reverse transcription complex (RTC) that is thought to arise as a result of viral uncoating [70].

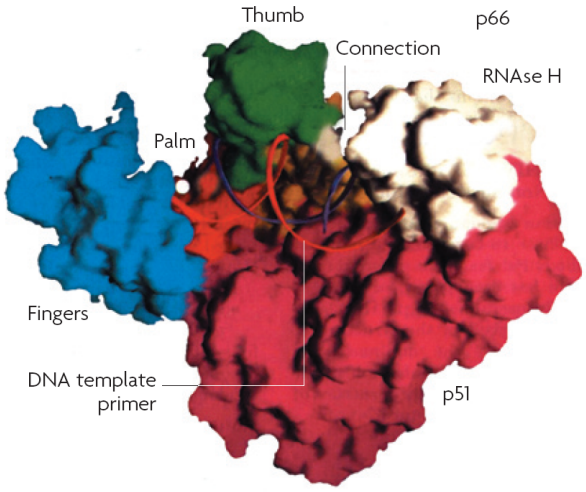


FIG 6 Structure of the HIV-1 reverse transcriptase

The picture shows the structure of the p51/ p66 reverse transcriptase heterodimer with a DNA template. The structure is a typical right handed polymerase structure with thumb, palm and finger domains. The connection and RNaseH domains are indicated. Figure derived from [74].

The detailed molecular process of reverse transcription is depicted in FIG 7. Reverse transcription (RT) is mediated by the viral reverse transcriptase, an asymmetric p51/ p66 heterodimer, which possesses two different catalytic activities, a DNA polymerase and a RNaseH activity for RNA digestion of RNA:DNA hybrids (FIG 6). The catalytic sites for both activities are found in the p66 form and the p51 appears to be structurally important.

Reverse transcription is initiated at the 3' end of a tRNA bound to the primer-binding site (PBS) located close to the 5' end of the HIV-1 RNA (FIG 7). For HIV-1 it is the $tRNA_{Lys3}$ that binds to the PBS and acts as a primer for reverse transcriptase. Selective packaging of $tRNA_{Lys3}$ into budding virions is likely to be mediated by the lysyl-tRNA

synthetase that binds $tRNA_{Lys3}$ and interacts directly with Gag during virus assembly [75]. The enzyme reverse transcriptase first synthesises complementary DNA of negative polarity (-) towards the 5' end of the RNA transcribing the RU5 region. During this process the RNaseH enzyme activity of the reverse transcriptase degrades the RNA strand of the newly generated RNA-DNA hybrid causing a single strand 3' DNA RU5 overhang also called (-) strong-stop DNA. The repeat region (R) of this overhang is able to anneal to the complementary 3' RNA repeat region either of the same molecule or with the second packaged RNA molecule. This strand exchange provides the virus with the ability to recombine. Annealing of the synthesised DNA to the 3' RNA R region serves to prime the RNA dependent DNA synthesis (RDDP). Elongation continues until it reaches the 5' RNA PBS. The (+) strand RNA in the DNA-RNA hybrid is then degraded by the RNaseH activity of reverse transcriptase, however a region close to the 3' RNA end possessing a ~11 nt long polypurine tract (PPT) escapes this degradation and functions as a priming site for the DNA-dependent polymerase activity of the reverse transcriptase (DDDP). This leads to the synthesis of the 3' end of the (+) DNA strand including the entire U3RU5 region as well as 18 nt of the tRNA sequence that is complementary to the PBS. In addition, HIV-1 has a second, more central, PPT region also mediating (+) DNA synthesis. The positive strand DNA synthesis stops when reverse transcriptase encounters a modified base in the tRNA sequence. The generated (+) DNA sequence that is complementary to the 3' end of the bound tRNA is called (+) strong-stop DNA. RNaseH activity degrades the tRNA primer and the RNA PPT causing a single stranded 3' (+) DNA overhang of the PBS. This sequence can anneal with the complementary PBS of the (-) strand DNA causing the second "strand exchange" and thereby creating a circular intermediate form of viral DNA. A linear double stranded DNA molecule with two identical LTRs is synthesised by the reverse transcriptase by strand displacement. During this process an aberrant circularised form with a single LTR (1-LTR circle) can form (FIG 3). Furthermore, ends of the linear DNA molecule can be joined by cellular DNA repair enzymes of the non-homologous end joining pathway, creating a second circular form denoted as 2-LTR circles (FIG 3). The formation of 2-LTR circles is seen as a marker for nuclear entry, since the ligases involved, Ku70 and Ku80, are nuclear enzymes [76].

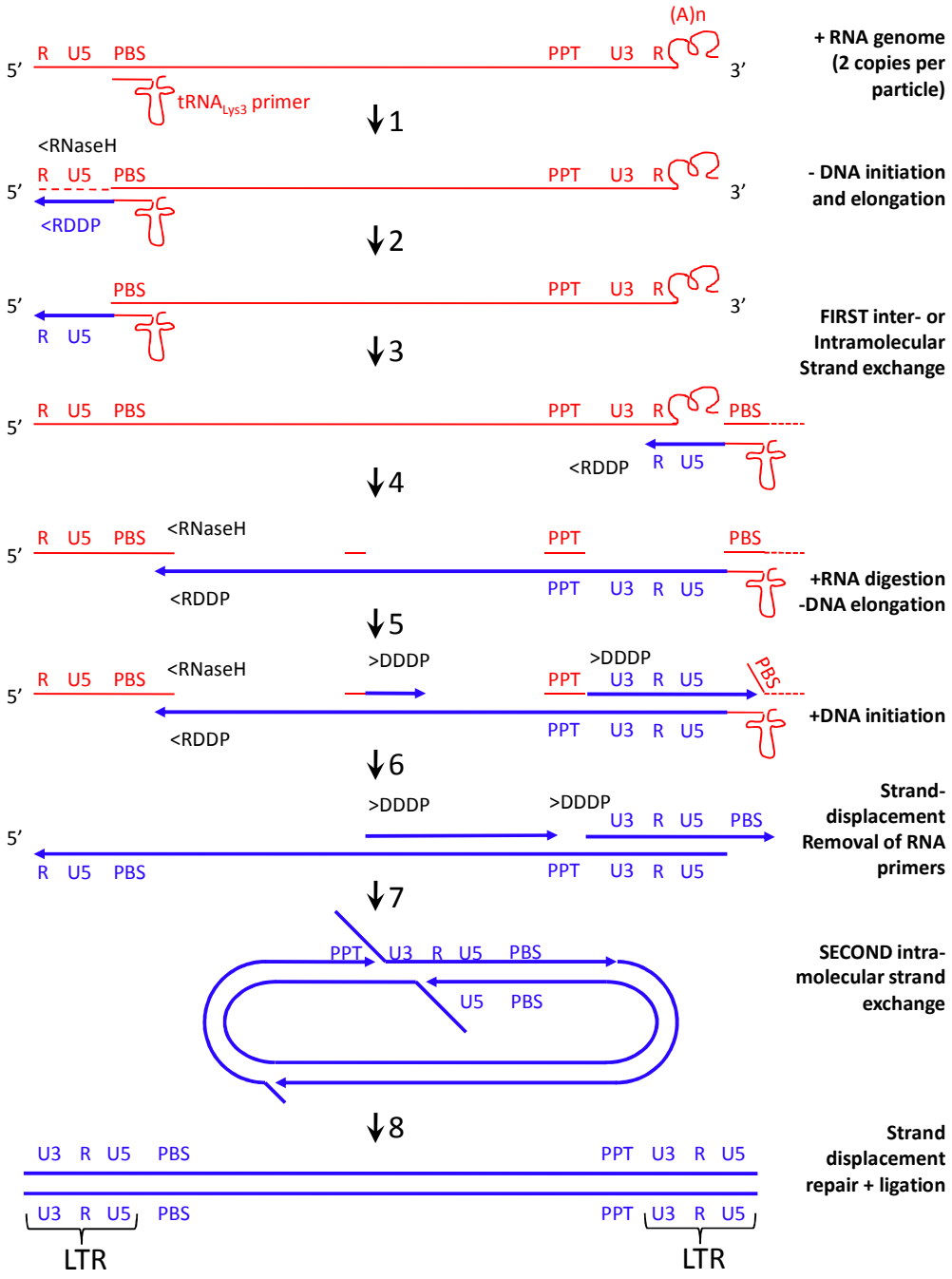


FIG 7 The process of reverse transcription of HIV-1

The HIV-1 RNA is reverse transcribed into DNA by the RNA dependent polymerase (RDDP) activity of the reverse transcriptase starting at the 3' end of tRNA primer. The 5' RU5 is transcribed and the RNA is degraded by the RNaseH activity of the reverse transcriptase (step 1 and 2). The first inter- or intramolecular strand exchange (sometimes referred to as the first jump) leads to the initiation of reverse transcription of the negative DNA strand from the 3'R up to the PBS in the 5' RNA region (steps 3 - 5). A polypurine tract (PPT) initiates the DNA dependent polymerisation (DDDP) of the positive DNA strand (step 5-6). Additional initiation of positive DNA strand synthesis occurs along the negative DNA strand at a central PPT. In the second strand exchange (second jump), the 3' PBS of the negative DNA strand anneals with the 3' PBS of the positive DNA strand and DDDP occurs via strand displacement. Linearised double stranded viral DNA with two identical LTRs, one at each end, is able to integrate into the host genome. In addition, ligation of the linear genome before integration can lead to 2-LTR circle formation and recombination can create 1-LTR circles (not shown).

1.4.3. Nuclear import of HIV-1 PICs

To integrate their genomes successfully into the host, retroviruses need to enter the nucleus of an infected cell (FIG 3). In contrast to MLV, HIV-1 is able to infect non-dividing cells but the molecular details underlying this difference remain poorly understood [77]. Studies have shown that RTCs from MLV contain capsid (CA), integrase (IN) and reverse transcriptase proteins, whereas CA was not detected in HIV-1 RTCs [70, 73, 78]. HIV-1 RTCs also contain the viral protein Vpr, as well as matrix (MA), reverse transcriptase and integrase [70, 79]. The absence of CA protein in cell fractions of HIV-1 infected cells with high reverse transcriptase activity as well as the inability to immunoprecipitate viral DNA with anti-CA antibodies suggests that if at all, very low numbers of CA protein are associated with HIV-1 RTCs [79]. In contrast, more recent studies suggested that the HIV-1 CA protein may be directly involved in the process of infecting non-dividing cells at a step past nuclear entry. For example, a study using HIV-1, in which the MA-CA coding region was replaced by MLV MA-p12-CA, showed that the chimaeric virus was inhibited in infecting aphidicolin arrested cells [80]. Replacement of the HIV-1 MA by MLV MA-p12 however had no effect on infecting non-dividing cells [80]. Furthermore, CA mutations altering cyclophilin A sensitivity of HIV-1 seem to affect the ability of the virus to infect non-dividing cells [81]. These observations imply that at least some CA proteins may be present in the pre-integration complex (PIC) entering the nucleus [72].

Every viral protein present in the HIV-1 PIC, apart from reverse transcriptase, has been proposed to be involved in mediating nuclear entry. The first protein to be implicated in nuclear import via two different internal nuclear localisation signals (NLS) was the matrix protein (MA/ p17) [82, 83]. However, tagged MA protein is not localised to the nucleus [84] and pseudotyped HIV-1 particles with a non-functional MA are still able to infect macrophages [85], suggesting that the matrix protein is dispensable for nuclear import of PICs.

Vpr has been suggested to mediate HIV-1 PIC nuclear import in macrophages due to its karyophilic property and by binding to members of the importin alpha family [86, 87]. Further studies showed that Vpr has two NLS motifs [88]. Moreover, *in vitro* studies using digitonin-permeabilised cells suggested that Vpr is the key regulator for nuclear import of HIV-1 PICs. These studies demonstrated that Vpr mediates the docking of the PICs to nuclear pore complexes (NPC) and that recombinant Vpr provided in *trans* could restore 2-LTR circle formation of Vpr defective virus, suggesting

complementation of nuclear import [89, 90]. An unconventional mechanism of Vpr function proposed to promote HIV-1 infection in arrested cells is the herniation of the nuclear envelope [91]. The importance of MA and Vpr in HIV-1 infection remains controversial and several reports have shown that disruption of nuclear localisation of MA and Vpr is insufficient to abolish infection of cell cycle arrested cells or primary macrophages [92, 93]. Evidence that a third factor is involved in mediating nuclear import in non-dividing cells came from a study in which high doses of HIV-1 with a NLS deleted MA and without Vpr were used to infect terminally differentiated macrophages. The data showed that high virus doses could overcome the defect caused by the deletions [94]. However, additional mutation of the HIV-1 integrase abolished nuclear translocation suggesting a role for this protein in nuclear entry. The integrase mediated import was dependent on the karyopherin alpha import pathway [95].

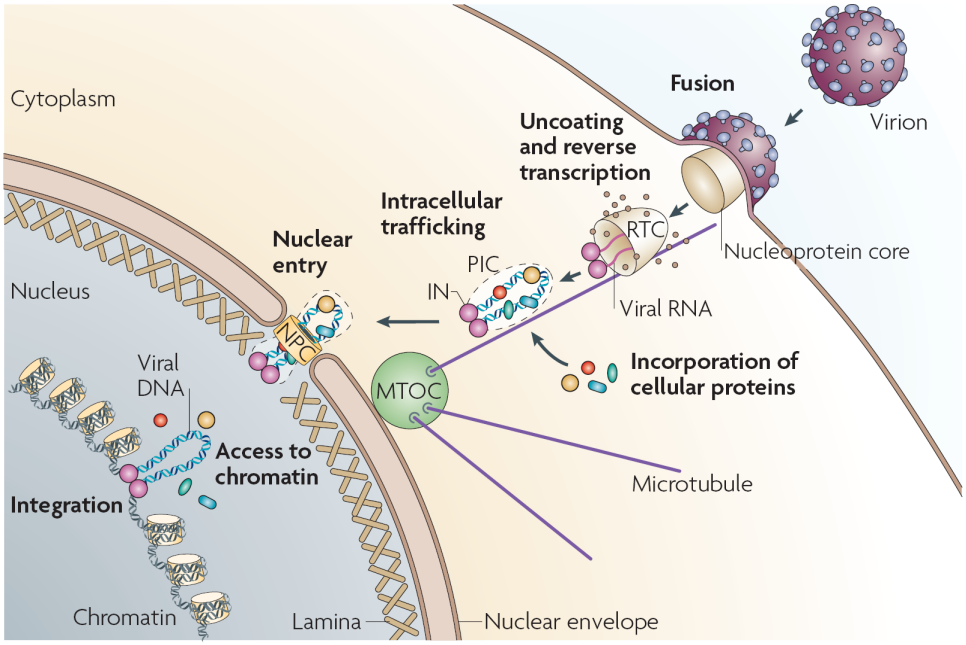


FIG 8 Nuclear import of HIV-1 pre-integration complexes (PICs)

A) Pathway of HIV-1 in a cell shortly after infection. Virions fuse with the plasma membrane and the core is delivered into the cytoplasm. Uncoating releases viral RNA which is reverse transcribed into DNA in a complex with proteins (RTC). After synthesis of viral DNA the pre-integration complex (PIC) is formed that contains at least matrix, Vpr, integrase and reverse transcriptase and probably multiple cellular factors (e.g. LEDGF/ p75). Intracellular trafficking of RTCs and PICs towards the nucleus appears to be via microtubules [96]. Nuclear entry is believed to occur at the nuclear pore complex (NPC). Binding of LEDGF/ p75 to integrase in the cytoplasm may facilitate nuclear translocation of the PICs. Integration site specificity appears to be directed by LEDGF/ p75. MTOC, microtubule organisation centre. Figure derived from [97].

Recent data proposes a model in which integrase binding to the lens-epithelium-derived growth factor (LEDGF/ p75) (see 1.4.4.), a component of functional HIV-1 PICs, enhances their nuclear entry [98, 99].

In addition to MA, Vpr and IN proteins, the triple strand DNA structure found in the central polypurine tract (PPT) after reverse transcription referred to as the DNA flap, has also been reported to play a role in nuclear import [100]. Deleting or mutating this *cis* acting element, leads to the accumulation of linear unintegrated DNA at the vicinity of the nuclear membrane and significantly impairs nuclear import [100]. Interestingly, it has been suggested that the DNA flap promotes uncoating of HIV-1 at the nuclear pore [71]. In such a model the capsid shell is shed directly at the NPC followed by nuclear translocation of the PIC. However, like the whole field of nuclear import these observations are controversial and some work suggests wild type levels of HIV-1 replication in the absence of the central DNA flap [101]. Studies using an *in vitro* nuclear import assay have suggested that tRNAs with defective 3'CCA ends are transported into the nucleus in an energy and temperature dependent manner and can promote HIV-1 PIC nuclear import [102]. How this is achieved and the physiological relevance of this observation is currently unclear.

Early studies suggested that in primary macrophages HIV-1 PICs gain access to the nucleus using an active, RanGTP dependent transport mediated by importin-7 binding to integrase [103]. SiRNA mediated reduction of importin-7 levels however only reduces HIV-1 nuclear import by a few fold, suggesting that importin-7 plays a minor role. In addition, importin-7 is not involved in nuclear import of other lentiviruses including HIV-2, SIVmac and EIAV [104]. A genome wide siRNA screen aiming to identify host factors involved in the HIV-1 life cycle revealed the importance of the karyopherin transportin 3 (transportin-SR2/ TNPO3) for nuclear entry of HIV-1 but not MLV [105]. Furthermore, in analogy to a study on *Drosophila* homologues, this study suggested that the nuclear pore proteins Nup153 and Nup358/ RanBP2/ Cyp358 are involved in nuclear entry by interaction with TNPO3 [106]. In agreement with this prediction, TNPO3 and Nup358 also appeared in a second RNAi screen for HIV-1 co-factors [107]. TNPO3 was first identified in a yeast-two hybrid screen as a binding partner for HIV-1 IN and RNAi mediated reduction of TNPO3 reduced HIV-1 and HIV-2 replication by 3 to 4-fold [108]. Interestingly, the yeast orthologue of TNPO3, *MTR10*, seems to promote tRNA shuttling from the cytosol back to the nucleus [109].

The observation that some tRNAs enhance HIV-1 nuclear import may be connected to the function of TNPO3 in promoting HIV-1 nuclear import [102].

1.4.4. Integration of HIV-1 DNA into the host genome

For a productive infection the viral double stranded linear DNA molecule has to integrate into the host genome establishing a provirus (FIG 3). Integration occurs with the activity of the viral enzyme integrase (IN), p32 [110]. Recently, IN inhibitors have become available for treatment of HIV-1/AIDS and these are expected to complement protease and reverse transcriptase inhibitors.

HIV-1 IN is a 32 kDa protein that is derived from the Gag-Pol precursor. Integrase consists of an N-terminal zinc-binding domain that is likely to support multimerisation, a central catalytic domain and a C-terminal DNA binding domain, which associates non-specifically with DNA. Integrase promotes two distinct reactions that are necessary for virus DNA integration. The first reaction takes place in the cytosol and is the 3' processing in which a conserved *CA* dinucleotide is endonucleolytically liberated from the 3' ends of the double stranded viral DNA (FIG 9BII). Thereby, two reactive hydroxyl-groups are generated, which attack a phosphodiester bond in the host genomic DNA (FIG 9BIII). In the process of 3' end-joining integrase ligates the viral DNA into the host DNA, creating a single-stranded DNA segment of five base pairs at each end of the host DNA and a two nucleotide 5' viral DNA flap. The cellular DNA repair machinery fills the gaps and ligates the synthesised DNA into the host genome [111]. In addition to the 3' processing and 3' strand transfer reaction, two other reactions catalysed by integrase have been observed *in vitro*. The first reaction is a process referred to as disintegration and is the reverse of the 3' strand transfer reaction, however there is currently no data for its *in vivo* relevance. Second, tetrameric integrase complexes have been shown *in vitro* to cleave small oligonucleotides possessing the junction sequence between two LTRs that is seen in naturally occurring 2-LTR circles, suggesting that 2-LTR circles may not represent a dead end infection [112]. Interestingly, when integration is abolished for example by mutating integrase, 2-LTR circles are increased suggesting that this function may play a role in ensuring efficient integration *in vivo* [113]. HIV-1 does not integrate randomly into the host genome but prefers transcriptionally active sites [114]. To target sites for integration HIV-1 exploits specific host factors.

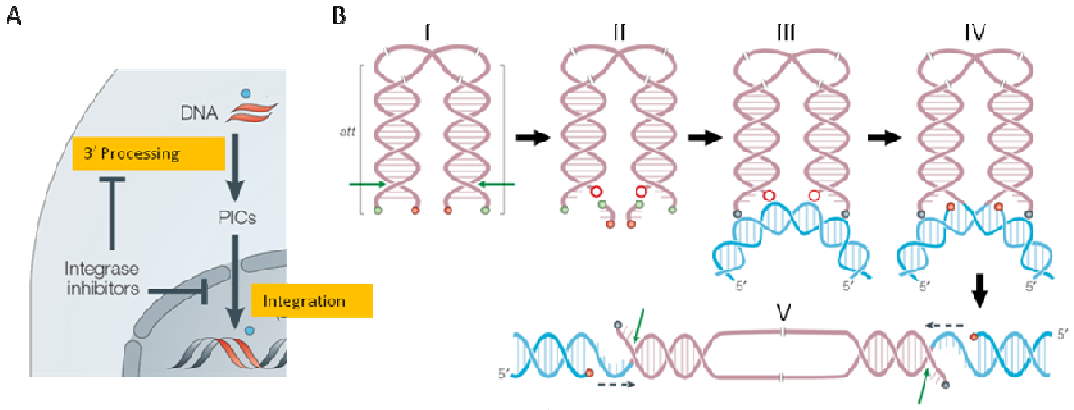


FIG 9 Integration of HIV-1 DNA into the cellular host genome

A) The process of integration of the HIV-1 DNA into the host genome is depicted. Double stranded viral DNA is processed by integrase and imported into the nucleus in the form of the pre-integration complex (PIC). Integration into the host genome creates the provirus. B) The viral DNA recombination sites (*att*) are depicted (I). Integrase creates a two-base pair 5' overhang at each site of the viral DNA by nucleolytic liberation of a conserved *CA* dinucleotide from the 3' end, a reaction called 3' processing (II). After nuclear import the generated hydroxyl groups at the 3' end attack a DNA phosphodiester bond in the host genome DNA in a process referred to as 3' end joining (IV). This leads to a five-base single-stranded sequence at each end of the host acceptor DNA and a two nucleotide 5' overhang (flap) of the integrating provirus. Host cell DNA repair enzymes generate double stranded DNA, and the 5' flap is cleaved off generating the fully integrated provirus (V). Figure adapted from [115].

Emerin, an integral inner-nuclear-envelope protein, has been proposed to be important for HIV-1 integration in macrophages [116]. A recent study investigating cells derived from emerin knockout mice however challenged this study and suggested that emerin and the protein lamina-associated polypeptide 2alpha (LAP2α) are dispensable for HIV-1 infection [117]. The barrier to auto-integration factor (BAF) and possibly the high-mobility group protein A1 (HMG-I(Y)/ HMGA1) may also be involved in integration of HIV-1 viral DNA. Purification of PICs and subsequent addition of cell fractions, initially identified HMGA1 to be important for HIV-1 infection [118]. A similar assay identified BAF binding to viral DNA, thereby facilitating inter-molecular integration and inhibiting auto-integration [119]. This study also investigated the impact of HMGA1 and bovine RNaseA, a non-specific DNA-binding control, and found that both proteins restored *in vitro* integration of purified PICs at very high concentration, suggesting that the function of HMGA1 is non-specific. BAF non-specifically binds double stranded DNA and co-immunoprecipitates with the HIV-1 proteins Gag and MA and may be packaged into virus at up to three copies per virion [120]. However, the exact mechanism of BAF function and its physiological relevance during infection is not understood.

Despite controversial observations in the early stages after the discovery of the integrase binding protein LEDGF/ p75, evidence for a functional role of this protein in enhancing and directing integration is increasing. LEDGF/ p75 binds integrases from HIV-1 and other lentiviruses but not from non-lentiviral retroviruses [99, 121]. SiRNA mediated reduction of LEDGF/ p75 expression or gene deletion in mice, decreases integration of HIV-1 and ablates the preference for transcriptionally active sites in the genome [122, 123]. Directed integration seems to be concerted by an interaction of LEDGF/ p75 with the HIV-1 IN because mutations in IN that affect LEDGF/ p75 interaction also impair HIV-1 integration [124]. Furthermore, a stable functional complex of dimeric LEDGF/ p75 and a tetrameric full-length integrase molecule could recently be reconstituted *in vitro*. This complex showed enhanced integration activity as compared to the integrase alone [125]. The study also revealed structural insights into the interaction of IN with LEDGF/ p75 and viral and host cell DNAs (FIG 10).

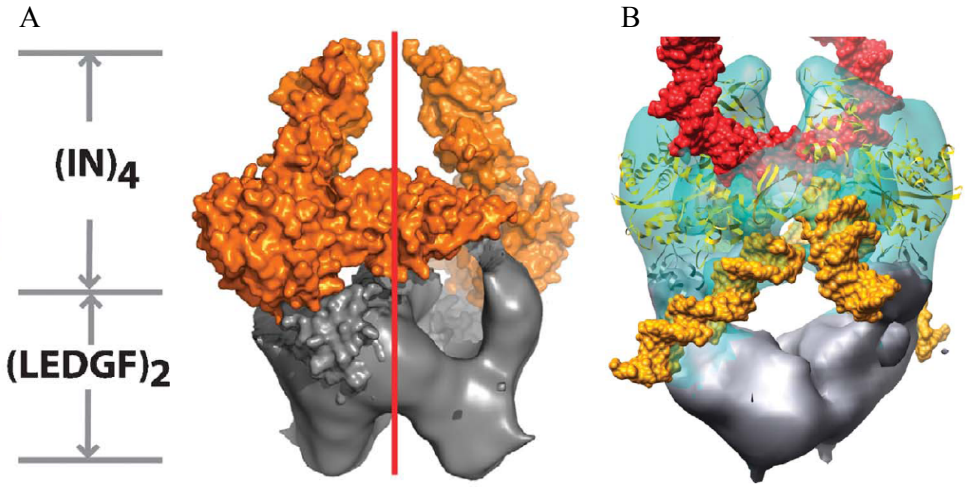


FIG 10 Structure of HIV-1 integrase bound to the cellular co-factor LEDGF/ p75

A) Depicted is the 3-dimensional structure of an HIV-1 integrase tetramer (orange) associated with a LEDGF/ p75 dimer (grey). B) The viral DNA (yellow) and the host cell target DNA (red) are shown before 3' strand transfer reaction. Figure derived from [125].

1.4.5. Transcription and the function of Tat

After the virus has established a provirus, transcription of HIV-1 is mediated by the cellular RNA polymerase II (RNA Pol II) in a similar way to the transcription of cellular host genes. HIV-1 transcription occurs in two distinct phases, an early phase independent of transactivation and a late phase that depends on the presence of the viral protein Tat. Transactivation of transcription by Tat is an important step in the HIV-1 life cycle (FIG 11). A region located between nucleotides +1 and +59 downstream of the HIV-1 LTR transcription start site forms the transactivating responsive region (TAR) in synthesised RNAs [126, 127]. During transcription the RNA adopts a stem loop structure in this region. RNA Pol II transcription elongation is mediated through Tat binding to TAR and the recruitment of the cellular transcription elongation factor P-TEFb [128]. P-TEFb consists of CyclinT1 and the cell cycle dependent kinase 9 (Cdk9) that mediates the hyperphosphorylation of the C-terminal domain (CTD) of the RNA Pol II [129]. Hyperphosphorylation of the CTD leads to efficient transcription elongation by RNA Pol II. Efficient transcription of the HIV-1 provirus requires the binding of different transcription factors upstream of the transcription start site, e.g. NF-κB and Sp1 (FIG 11). In addition to the well studied activity of Tat to initiate transcription elongation with the help of P-TEFb by binding to the TAR stem loop, Tat may also enhance the assembly of the transcription pre-initiation complex by promoting TATA-binding protein (TBP) association with the TATA box [130]. The positive effect was observed for TBP recruitment to the TATA box, however an increase in TBP-associated factors (TAFs) was not observed [130]. Interestingly, whilst Tat transactivates HIV-1 transcription it also can have a repressive effect on transcription of cellular genes (e.g. MHC class I genes) by binding to TAFII250 and inhibiting its histone acetyl transferase activity [131]. In addition, Tat functions to inhibit repressors of HIV-1 transcription like the cleavage and polyadenylation specificity factor (CPSF) complex that has been demonstrated to bind and inhibit the HIV-1 LTR promoter [132]. In fact, Tat interacts with a variety of factors involved in transcriptional control but the importance of these interactions remains unclear.

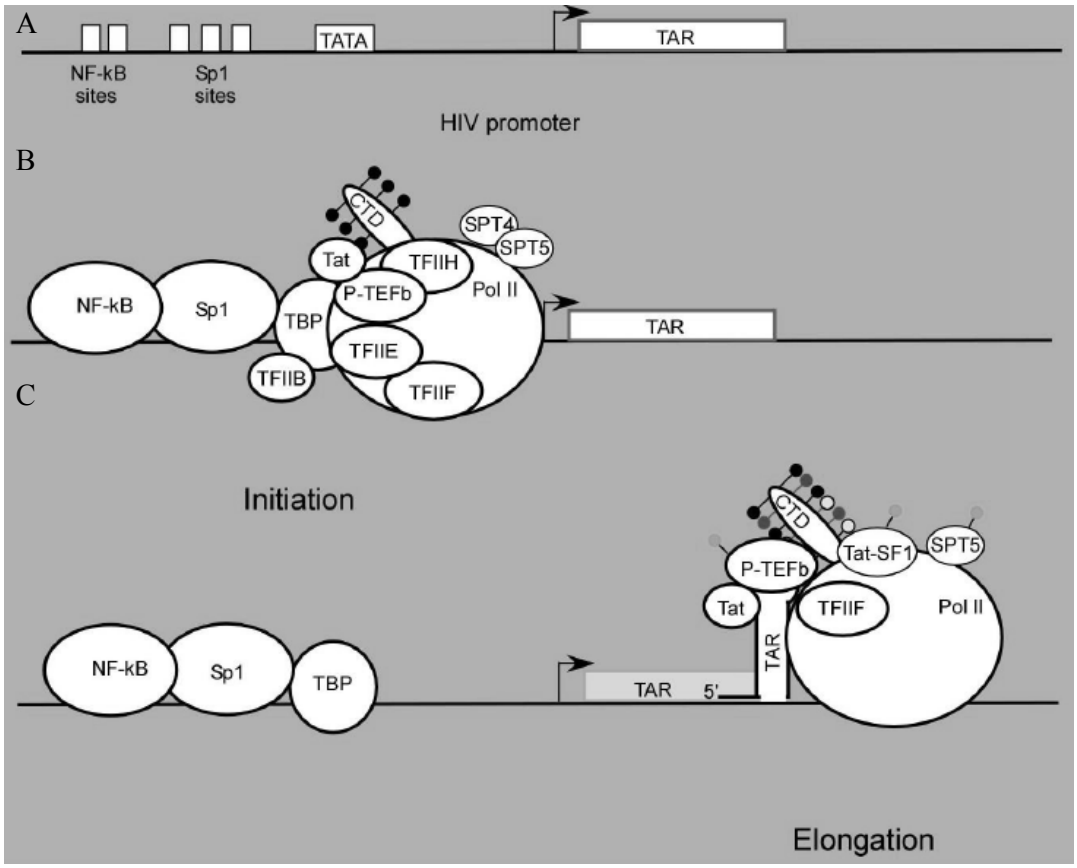


FIG 11 Transactivation of transcription by HIV-1 Tat

A) The HIV-1 LTR promoter is shown. Upstream of the transcription initiation site NF-κB and Sp1 binding sites as well as a TATA box can be found. Downstream of the transcription start site the transactivating-responsive region (TAR) is located. B) HIV-1 transcription initiation occurs by NF-κB and Sp1 binding and assembly of the large RNA polymerase II complex at the TATA-binding protein (TBP). Tat binds to P-TEFb, a transcription elongation factor consisting of cyclinT1 and cdk9, which itself associates with the RNA Pol II transcription initiation complex. C) Transcription is stalled after TAR has been transcribed until Tat binding to both P-TEFb and TAR initiates P-TEFb dependent hyperphosphorylation of the C-terminal domain of RNA Pol II promoting transcription elongation. Figure adapted from [133].

The HIV-1 provirus is established in the context of host cell chromatin. Immediately up-/ and downstream of the integration site nucleosomes are formed, repressing HIV-1 transcription. To ensure efficient transcription, chromatin-remodelling enzymes have to be recruited that modify the downstream nucleosome. Tat recruits histone acetyltransferase proteins including p300/CREB-binding-protein (CBP), the p300/CBP-associated factor (PCAF) and the general control nonderepressible 5 protein (GCN5) [134, 135]. Tat itself is the target for modifications by PCAF and GCN5, and Lys-28 acetylation of Tat has recently been demonstrated to be important for stabilising the TAR-Tat-P-TEFb complex [136]. Furthermore, Tat is also subject to deacetylation by SIRT1, which may be a way to recycle acetylated Tat [137]. In addition to acetylation

of Tat, acetylation of the N-termini of histone proteins H3 and H4 located in the nucleosome downstream of the transcription initiation site enhances HIV-1 provirus transcription. Further evidence for chromatin-remodelling to ensure efficient HIV-1 transcription is the recruitment of the ATP-dependent SWI/SNF chromatin-remodelling complex that appears to be a co-factor for Tat transactivation of transcription [138].

1.4.6. Rev dependent export of partially spliced and unspliced mRNAs

To ensure the production of virus proteins, mRNAs synthesised in the nucleus need to be exported into the cytoplasm. Molecular interaction partners for mRNA export are depicted in FIG 12. Cellular mRNAs are exported with the help of the two proteins Tap and Nxt1, which bind mRNA in the nucleus as a heterodimer. Cellular mRNAs bind to the host cell protein UAP56, which associates with the protein Aly that in turn recruits the Tap/ Nxt1 heterodimer [139]. Nuclear mRNAs are therefore loaded with a variety of proteins before they exit the nucleus to ensure correct processing and transport, and to prevent the export of unspliced or partially spliced mRNAs. For efficient nuclear mRNA export, a process of remodelling during which the bound proteins dissociate from the mRNA is important. Cellular mRNAs are bound by the DEAD box helicase Dbp5, which interacts with proteins of the NPC and mediates translocation and remodelling of the mRNA as well as subsequent translation termination [140, 141]. Some simple retroviruses like Mason Pfizer monkey virus (MPMV) exploit this pathway by directly recruiting Tap/ Nxt1 to their constitutive transport element (CTE), a *cis* acting sequence found in their unspliced mRNAs.

In contrast to most cellular mRNAs, HIV-1 generates multiple singly spliced and unspliced mRNAs (FIG 13). The HIV-1 early transcripts Tat and Rev, as well as Nef are multiply spliced and the processed mRNAs are exported from the nucleus into the cytoplasm via the cellular mRNA export pathway. The HIV-1 Rev and Tat protein are both imported into the nucleus after their synthesis. The export of unspliced full-length viral RNAs, that encode the Gag and GagPol polyproteins or serve as genome RNA that is packaged into virions, is dependent on the viral Rev protein. Viral mRNAs encoding Env and Vpu, Vif, Vpr and Tat are singly spliced, and their nuclear export is also dependent on Rev. Rev binds to the Rev-responsive-element (RRE), a very conserved *cis* acting region in the Env coding region (FIG 2) that forms a strong secondary structure with several stem loops [142]. Rev binds the stem-loop IIB, initially as a

monomer followed by further association of Rev monomers through Rev-Rev interactions [143]. Oligomerisation of Rev at the RRE has been suggested to be important for its function and as many as eight molecules can bind to the RRE [144, 145]. Since Rev's interaction with the RRE is essential for nuclear export of HIV-1 RNAs, it presents a suitable target for drug development. A dominant negative Rev protein, RevM10, has been investigated as a suitable drug. However, virus escape mutants arise by changing their RRE conformation [146, 147].

Partially or unspliced cellular mRNAs are bound by splicing commitment factors in the nucleus, which cause their nuclear retention in the Tap/ Nxt1 pathway. To circumvent this block of singly- or unspliced viral RNA export, HIV-1 uses a different export pathway involving the cellular proteins chromosome region maintenance 1 (Crm1) and the DEAD box RNA helicase DDX3. In contrast to the Tap/Nxt pathway, Crm1 mediated export is dependent on the GTPase Ran and can specifically be blocked by the drug leptomycin B. The Crm1 pathway is usually used to export proteins, small nuclear RNAs and ribosomal RNAs and is distinct from the pathway used for completely spliced HIV-1 RNAs. In the nucleus Crm1 associates with RanGTP and replaces the eukaryotic initiation factor 5A (eIF5A) that is bound to the viral Rev protein [148]. The Rev protein contains a nuclear export sequence (NES) that is recognised by Crm1. In addition, DDX3 binds to the RNA/Rev/Crm1/RanGTP complex as well as to proteins of the NPC, suggesting that it has a similar role to Dbp5 to support RNA remodelling after or during nuclear export [149]. The nuclear RNA/Rev/Crm1/DDX3/RanGTP complex associates with FG repeat nucleoporin proteins at the NPC, mediating the export of the complex. RanGAP-mediated RanGTP hydrolysis in the cytoplasm leads to the disassembly of the complex and initiates subsequent steps of RNA translation or transport of genomic RNA for packaging into virions. Rev and Crm1 cycle between the cytoplasm and nucleus so that a constant export of viral RNAs is ensured. Genomic RNAs are translated into the Gag and GagPol polypeptides or packaged as non-covalently linked dimers into newly forming virions. Packaging is achieved by the interaction of a specific nucleocapsid (NC) protein domain in Gag with the *cis* acting Ψ site located near the 5' end of the genomic RNA. Dimerisation of the genomic RNAs is important for efficient packaging and generation of infectious HIV-1 [150]. However, the subcellular site for HIV-1 genomic RNA dimerisation is not known.

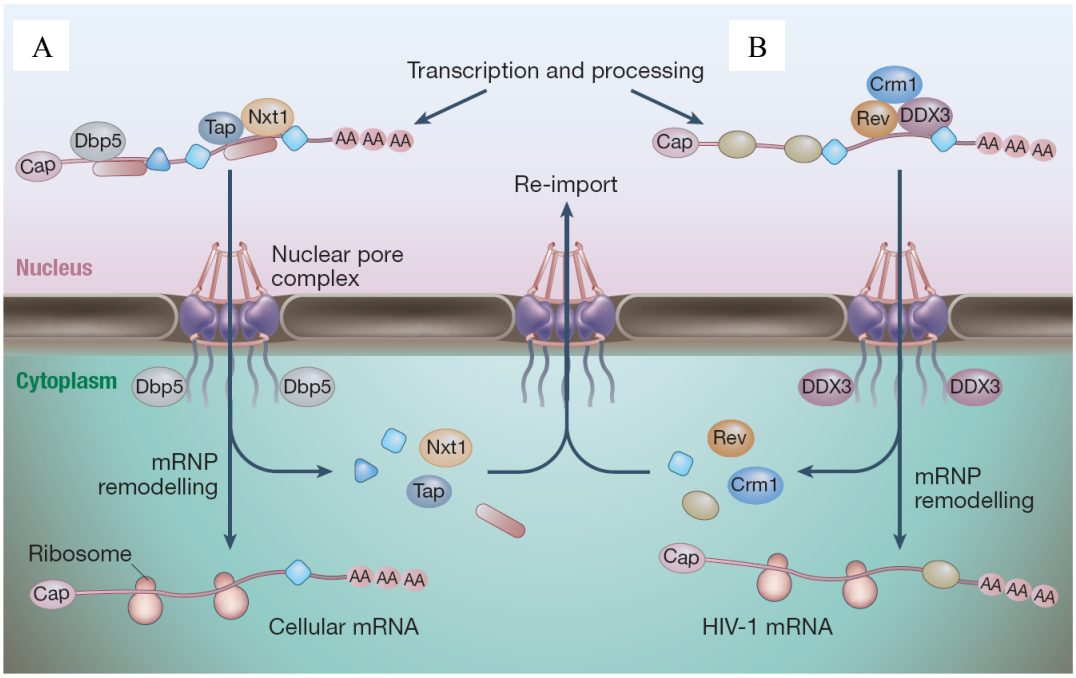


FIG 12 HIV-1 nuclear RNA export compared to the cellular mRNA export pathway

A) Transcribed and correctly spliced cellular mRNAs are exported from the nucleus by binding of TAP and Nxt1. B) The HIV-1 Rev protein binds viral RNAs at the RRE *cis*-sequence and recruits Crm1 and DDX3, which mediate nuclear export of singly and unspliced RNAs. Figure derived from [151].

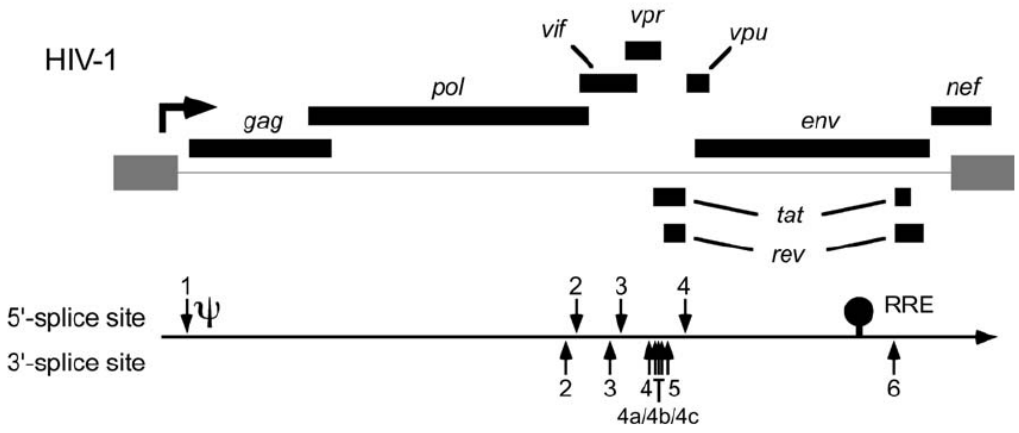


FIG 13 Splicing of the HIV-1 RNA

HIV-1 splice sites are shown. HIV-1 has got at least four different 5' splice sites and at least eight different splice acceptor sites (3' splice sites). Partially spliced mRNAs and unspliced genomic RNA have the RRE *cis*-element and are exported in a Rev dependent manner, whereas Tat, Rev and Nef encoding mRNAs are exported in a Rev independent way. Figure derived from [152].

1.4.7. Gag trafficking and HIV-1 budding via the cellular ESCRT machinery

To complete their life cycle, viruses need to ensure the efficient production and release of infectious virus particles. For the generation of virus particles the Gag protein has to be delivered to Env containing budding sites at the plasma membrane.

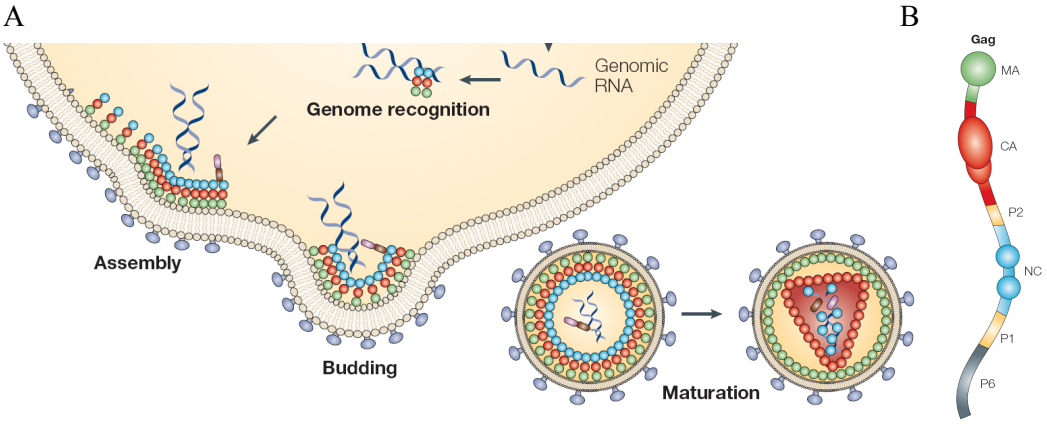


FIG 14 Gag interaction with a genomic RNA dimer and trafficking

A) Synthesis of Gag and GagPol leads to accumulation of the proteins at the plasma membrane and interaction with a dimer of genomic viral RNAs mediating its packaging into newly formed virions. B) HIV-1 Gag consists of matrix (MA), capsid (CA), p2, nucleocapsid (NC), p1 and p6 and multimerisation involves domains in MA, CA-p2 and NC. Figure derived from [153].

Newly synthesised Gag and GagPol proteins are co-translationally modified with the 14-carbon fatty acid myristate. Myristyolation occurs at the N-terminus of the MA protein and this modification is important for membrane association of Gag as well as particle formation. Membrane association of Gag is induced by the exposure of a myristate group in MA [154]. Gag multimerisation at the plasma membrane is mediated by regions in NC, CA-p2 and MA [155]. HIV-1 Gag proteins associate at the plasma membrane with lipid microdomains, called lipid rafts. Gag trafficking to the plasma membrane may involve intermediate compartments in cells defined as multivesicular bodies (MVBs). Studies have shown that AP-1, AP-2 and AP-3, members of the clathrin adaptor protein (AP) complexes interact with Gag. For example, AP-3 has been suggested to be important for Gag trafficking from MVBs to sites of assembly at the plasma membrane. AP-3 depletion caused Gag accumulation in MVBs and aborted infectious particle production [156-158]. Gag localisation to the plasma membrane may also be sensitive to phoshpatidylinositol-4,5 biphosphate (PIP_2) levels [159]. When a constitutively active form of the cellular protein Arf6 was expressed, endosomal structures with a high content of PIP_2 were formed and virions budded into these

compartments, suggesting a similar role for plasma membrane associated PIP_2 in direct Gag recruitment [159].

HP68 is a protein that has been proposed to be involved in the formation of the Gag core during virus assembly [160]. It has been suggested that HP68 binds to Gag at virus assembly sites and its dissociation from Gag is important for the following steps in virus assembly [161]. Another protein that may be involved in virus budding is TIP47. It has been suggested to be required for Gag association with Env at virus budding sites and to be involved in Env trafficking [162, 163]. However, since this initial report no further results on its function in HIV-1 budding have been published.

Some membrane associated HIV-1 Gag is ubiquitinated and Gag ubiquitination is important for virus budding [164, 165]. Ubiquitination of Gag is highly dependent on its membrane association but the ubiquitin ligase mediating Gag ubiquitination has yet to be identified [165]. Nedd4-like E3 ubiquitin ligases may be involved in HIV-1 Gag ubiquitination since some members associate with retroviral Gag proteins [166]. The Nedd4-like E3 ubiquitin ligases may link Gag ubiquitination with the recruitment of the budding machinery described below. In addition, de-ubiquitinating enzymes like the associated molecule with the SH3 domain of STAM (AMSH) may be involved in regulating Gag ubiquitination at sites of virus assembly [167].

Retroviruses, including HIV-1, exploit the cellular endosomal sorting complex required for transport (ESCRT) machinery, which is comprised of three multi-subunit protein complexes ESCRT-I, II and III. Each of these complexes consists of a variety of different ESCRT proteins (FIG 15). The ESCRT machinery was first described in yeast as an important component for sorting of cargo into the yeast vacuole. This work has been translated to mammalian cell biology and important roles have been found for cargo sorting into MVBs, abscission during cell division and the budding of enveloped viruses at the plasma membrane. HIV-1 and other retroviruses possess short sequence motifs in their Gag proteins, which are called late domains (L-domains) and function to regulate virus budding by recruiting the ESCRT machinery. Mutation of these domains leads to the accumulation of virions that are unable to complete budding and are connected to the cell membrane by thin membrane stalks [168]. HIV-1 possesses multiple L-domains in its p6 protein, located at the C-terminus of Gag (FIG 2 and FIG 15). The PTAP motif found in the N-terminus of p6 binds the ubiquitin E2 variant (UEV) domain of tumour susceptible gene 101 (Tsg101), whereas the LYPXL motif in the C-terminus of p6 recruits the V-domain of ALG-2 (apoptosis-linked gene

2)-interacting protein X (Alix) [169-172]. Tsg101 and Alix are key players in the assembly and function of the ESCRT machinery and recruit ESCRT-I and ESCRT-III complexes, respectively. SiRNA mediated depletion of either of these two important proteins, or expression of dominant negative versions of proteins involved in the budding pathway, e.g. Vps4, results in a virus budding phenotype that is indistinguishable from viruses mutated in their L-domains [170]. Moreover, overexpression of Alix can rescue HIV-1 that is defective in recruiting Tsg101 due to the disruption of its PTAP motif and this rescue is independent of Tsg101 expression. This suggests that Alix's function for wild type virus budding may be of redundant nature and not via the ESCRT-I machinery [173]. Furthermore, Alix also binds to the HIV-1 nucleocapsid via its BroI domain, which was suggested to be important for budding [174, 175]. In addition, budding of PTAP or LYPXL mutated HIV-1 can be rescued by overexpression of a naturally occurring N-terminally truncated isoform of the Nedd4 E3 ubiquitin ligase called Nedd4L/Nedd4-2 or Nedd4-2s [166, 173]. This isoform binds to a region in HIV-1 Gag localised to the C-terminus of CA and the p2 protein. The exact binding site and the molecular mechanism however remain to be discovered, but the rescue seems to involve the assembly of the ESCRT-I machinery since Tsg101 reduction reduced the effect [173]. Alix recruits the ESCRT-III complex, which consists of a number of Chmp proteins that mediate the final step of membrane scission. After the virion has budded from the plasma membrane the ESCRT-III machinery is disassembled by the Chmp dependent recruitment of Vps4, a member of the AAA-ATPases, associated with a variety of activities.

The subcellular site of HIV-1 assembly has been very much discussed over the last years. In primary macrophages HIV-1 was suggested to assemble into cellular compartments, which showed markers of late endosomes [176]. Later studies however showed that this compartment is structurally a pre-existing invagination of the plasma membrane that may present a special compartment in macrophages [177]. Contradicting results were obtained however by other labs, showing that also in macrophages HIV-1 assembled at the plasma membrane [178]. The use of L-domain mutants in macrophages may help to elucidate the real site of assembly, however these experiments have yet to be performed.

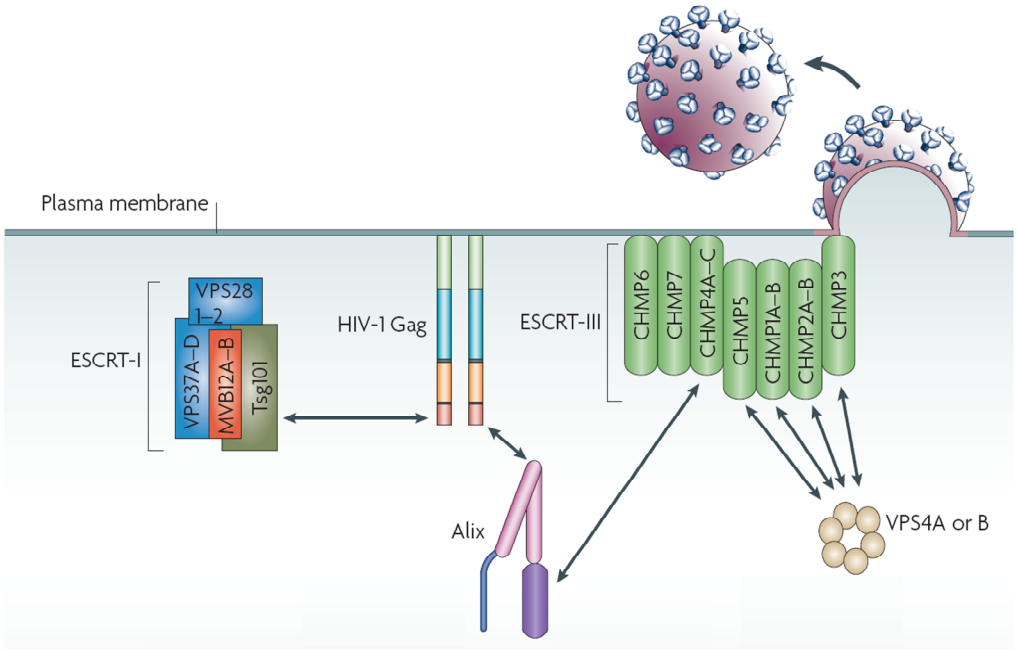


FIG 15 The ESCRT machinery and budding of HIV-1

The steps occurring at the plasma membrane before HIV-1 budding are shown. ESCRT-I is recruited to Gag by interaction of Tsg101 with the PTAP motif in HIV-1 Gag p6. Alix interacts with the LYPXL motif in p6 and recruits the ESCRT-III machinery via interaction with Chmp4 proteins. Chmp proteins are essential for the membrane scission and liberation of HIV-1 virions and recruit the ATPase Vps4. Figure adapted from [179].

1.4.8. Maturation

As soon as the virion has budded from the plasma membrane a process referred to as maturation takes place. The viral protease present in the few GagPol proteins that have been packaged into the virus cleaves Gag and GagPol proteins at distinct cleavage sites and liberates the proteins that constitute the internal structure of the virion, namely NC, CA, MA as well as the enzymes necessary for the next round of infection, integrase, protease and reverse transcriptase (FIG 16A and B). GagPol polyproteins arise when ~5% of the ribosomes translating Gag shift the reading frame at the end of Gag leading to further translation of Pol. Five different processing sites for the viral protease are located in Gag and all have been demonstrated to be essential for the infectivity of virions [180]. The order of cleavage is conserved starting with processing between SP1 and NC and ending with cleavage between CA and SP1 [181] (FIG 16C). Thus,

protease activity is the target for several drugs used in highly active antiretroviral therapy (HAART).

Liberated CA proteins form hexameric and pentameric fullerene structures, which build the cone shaped core of the mature virion (FIG 28A). Necessary for this structural maturation are conformational changes in the N- and C-terminus of cleaved CA. Nucleation of core assembly therefore depends on the correct cleavage by the virus protease.

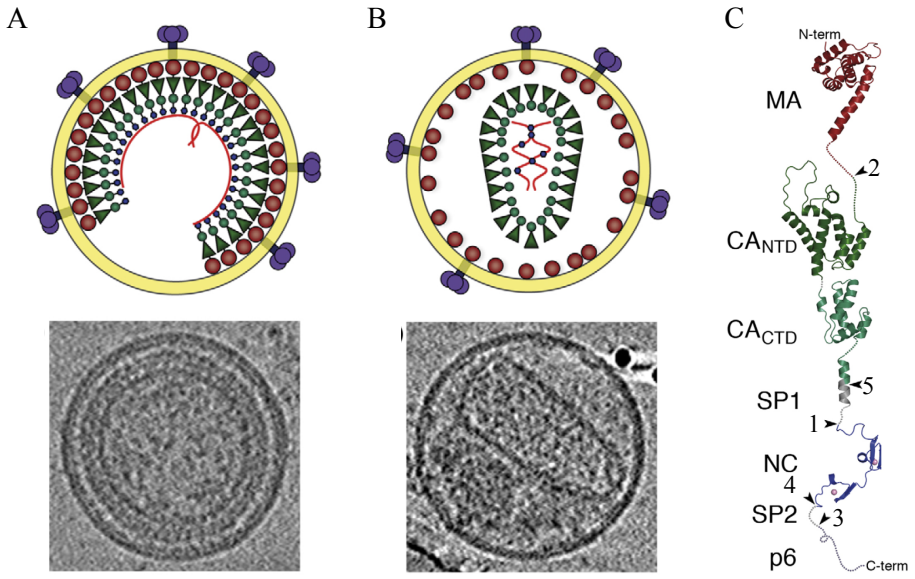


FIG 16 Maturation of HIV-1

A) After budding from the plasma membrane HIV-1 virions are immature and contain uncleaved Gag and GagPol proteins. The viral RNA dimer is associated with the NC domain of Gag. Internal cleavage events mediated by the viral protease lead to maturation of the virus. B) The mature virion contains a cone shaped core built of CA proteins burying the viral RNA dimer with attached NC proteins. MA proteins associate with the internal site of the viral membrane. Not shown are several other viral (Nef, Vpr) and cellular (e.g. CypA) proteins that also get packaged into the virions. C) Gag protein before cleavage. Protease cleavage sites are indicated with arrows. The numbers show the order of cleavage. Figure adapted from [180].

1.5. Innate immunity against retroviruses

Complex organisms have evolved many diverse ways to protect themselves from infection by pathogens, such as viruses or bacteria. The immune system is comprised of innate and adaptive arms and both are essential for efficient protection from pathogen infection. The innate immune system acts immediately after a virus has intruded an organism and includes for example the activation of the complement system and the production of interferons (IFNs), small molecules that are able to induce an antiviral state in the proximity of an infection. It is therefore an intracellular barrier that a virus encounters and it is important that it acts rapidly to limit virus replication and to provide time for the organism to induce the second arm of defence, the adaptive immune system. This acts via selection and expansion of T- and B-cells and the production of specific antibodies directed against the invading pathogen. The innate immune system of mammals also comprises a heterogeneous group of intracellular factors that are able to efficiently counteract viral infection in multiple ways. These factors, called restriction factors, constitute an intrinsic immune system, which counteracts an invading pathogen. In general these factors are interferon inducible and their expression is upregulated during early infection. They often act in a species-specific way, thus a restriction factor of one species usually does not strongly restrict viruses that infect the same species, however may block viruses from related species. Host and pathogen co-evolve, hence the evolution of each depends on the evolution of the other. The cell provides a restriction factor to act against a viral infection and the virus can evolve a countermeasure. Both, the restriction factor and the viral countermeasure provide a selective pressure on each other, often referred to as an evolutionary arms race. The antagonistic relationship between restriction factors and their viral countermeasures are often only poorly understood and the little information on the function and molecular mechanisms underlying restriction of viruses were mainly derived by studies on retroviruses. In this respect it is worth noting that some of the restriction factors are also attractive targets for drug development for diseases like AIDS [182].

Known retroviral restriction factors and viral countermeasures to bypass or inactivate these factors are depicted in FIG 17. A selection of these will be discussed in the following chapters.

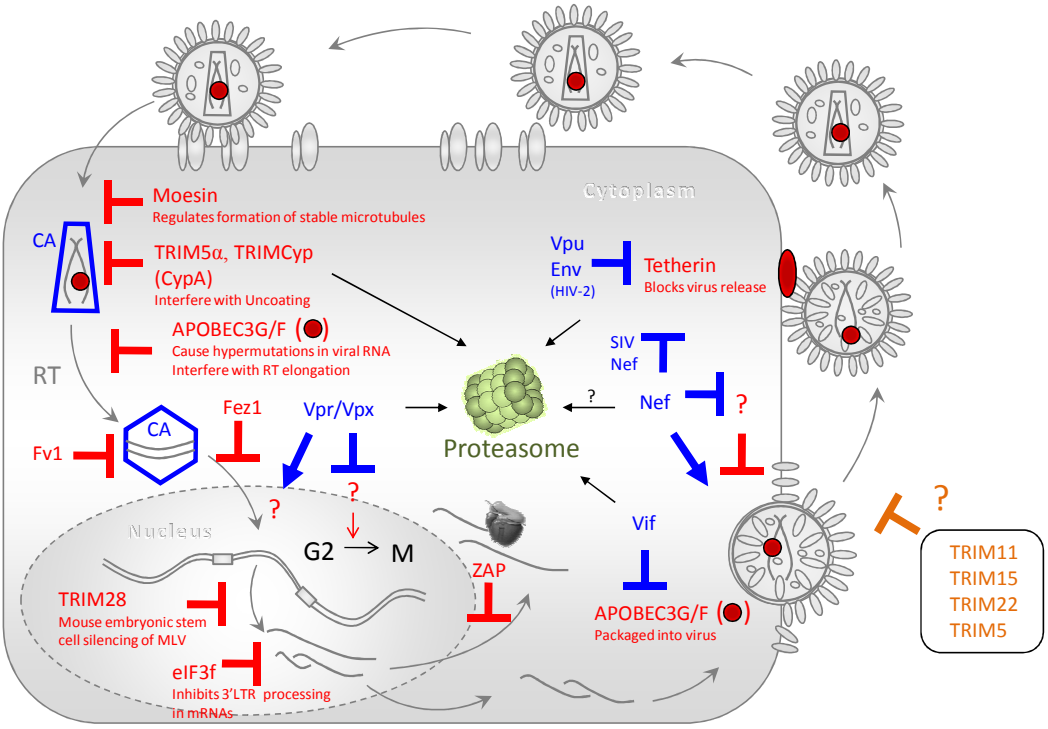


FIG 17 Host retroviral restriction factors and viral countermeasures

Cellular antiviral proteins are shown in red and retroviral countermeasures are shown in blue. Moesin regulates the formation of stable microtubules and blocks before initiation of reverse transcription [183]. Tripartite motif protein 5 isoform alpha (TRIM5α) restricts early infection by binding to CA structures of diverse retroviruses [184, 185]. Viruses escape restriction by changing CA residues (blue) interacting with TRIM5α. The TRIM5-cyclophilinA fusion proteins (TRIMCyp) found in owl monkeys and certain macaques bind to CA via their CypA domain [186, 187]. In addition, cyclophilin A levels impact on HIV-1 infectivity in a CA dependent manner [81]. Friend virus susceptibility 1 (Fv1) restricts certain MLV strains after reverse transcription but prior nuclear entry in a CA dependent manner [188]. Similarly, fasciculation and elongation protein ζ-1 (FEZ1) blocks retroviruses after reverse transcription but before nuclear entry [189]. Tripartite motif protein 28 (TRIM28) restricts MLV transcription in mouse embryonic stem cells by binding to the proviral primer-binding site [190]. The eukaryotic translation initiation factor 3 subunit f (eIF3f) inhibits the 3'LTR processing of viral mRNAs [191]. CCCH-type zinc finger protein ZAP depletes viral cytoplasmic mRNAs by an unknown mechanism [192]. Tetherin (Bst-2, CD317) inhibits release of diverse enveloped viruses and is counteracted by HIV-1 Vpu [193]. Nef from SIVs has also been suggested to block tetherin [194]. APOBEC3G/F proteins get packaged into HIV-1 in the absence of the viral Vif protein and cause hypermutations in the viral genome after infection, as well as inhibit elongation of RT in infected target cells [195-197]. HIV-1 Vpr supports nuclear import and causes cell cycle arrest in G2 possibly by binding an unknown factor, which may also be the target of the HIV-2/SIVsm Vpx protein. Nef increases infectivity of released virions possibly by inhibiting an unknown restriction factor in the virus producing cell, maybe in a way like Vif inhibits APOBEC3G/F. It also may act directly in a positive way via dynamin-2 [198]. Many viral proteins (Vif, Vpr, Vpx, Vpu) and some cellular factors (TRIM5, TRIMCyp) mediate degradation via the ubiquitin-conjugating and proteasome pathway, which becomes more and more a focus of intrinsic antiviral defence of cells.
Inhibitory roles of TRIM11 [199], TRIM15 [199], TRIM22 [200] and TRIM5 [201, 202] in HIV-1 production remain controversial.

1.6. The accessory viral proteins Vif, Vpr, Vpu and Nef

Simple retroviruses, e.g. MLV, encode the three major polyproteins Gag, Pol and Env. These three gene products are sufficient to ensure the successful life cycle of the virus. More complex retroviruses, e.g. lentiviruses, have evolved a subset of additional genes, referred to as accessory genes. Emerging data suggests that a major role of the HIV-1 accessory proteins Vpu, Vif, Vpr and Nef is to counteract innate and adaptive immune pathways or to modulate them to benefit the virus. Often they are dispensable for replication *in vitro*, but important *in vivo*.

1.6.1. HIV-1 Vpr causes cell cycle arrest

The HIV-1 protein Vpr has been briefly discussed above as a part of the pre-integration complex. It is involved in G2 cell cycle arrest, apoptosis and possibly transactivation of transcription and counteraction of an antiviral protein in macrophages. Vpr is a 96 amino acid long protein that is conserved across primate lentiviruses (FIG 2).

Vpr interacts with the first 46 amino acids of the p6 domain of Gag and is thereby packaged into virions [203]. However, Vpr was also found in isolated HIV-1 cores in the absence of p6 [204]. Vpr packaging has been used to study early events of HIV-1 infection by fusing a fluorophore onto Vpr [205]. Interestingly, in a recent study the fluorophore was replaced with a member of the APOBEC family, APOBEC3A, and this fusion protein showed an antiviral effect against HIV-1 and SIVmac upon infection of target cells [206]. In addition, Vpr fusion proteins with either chloramphenicol acetyltransferase enzyme or parts of Vpu reduced HIV-1 infectious titres up to 30 times compared to wild type HIV-1, suggesting that the increase of the protein size may impede virus production [207]. Vpr has also been suggested to interact with cyclophilin A (CypA), a cellular protein implicated in the permissivity of cells to HIV-1 infection (see 1.9.) [208]. It is possible that Vpr packaging into virions influences CypA packaging or vice versa but this has yet to be investigated.

Vpr expression leads to a G2 cell cycle arrest possibly by activating the G2 checkpoint ATM and Rad3-related kinase (ATR) (FIG 17) [209]. The physiological relevance of a cell cycle arrest has been suggested by the isolation of CA/p24+ CD4+ T-cells which were increased by up to 4-fold in a G2 arrested state with a tetraploid genome compared to CA/p24 negative CD4+ T-cells [210]. Studies trying to understand why HIV-1

manipulates the cell cycle showed that arresting cell division in G2 leads to an optimal HIV-1 LTR promoter activity and transcription [211]. Moreover, cell cycle arrest has been suggested to promote early steps of HIV-1 infection [212].

Furthermore, Vpr has recently been demonstrated to recruit a cullin-RING ubiquitin ligase, more precisely cullin4A-DDB1 [213, 214]. Vpr interaction with DDB1 is mediated via the receptor protein DCAF1, and siRNA mediated silencing of DCAF1 impairs the Vpr induced G2 cell cycle arrest [213]. Vpr binding to cullin4A-DDB1 seems to increase the activity of this E3 ligase [214]. Interestingly, DCAF1 was identified in a genome wide siRNA screen as an important factor for Vpr deficient HIV-1 infection [105]. Bringing these findings together a model suggests that Vpr enhances the activity of cullin4A-DDB1 to an unknown cellular substrate by forming a cullin4A-DDB1-DCAF1-Vpr complex, causing the cell cycle arrest [215]. A proposed alternative model is the recruitment of a cellular host factor by Vpr, causing its ubiquitination by the cullin4A-DDB1 complex and degradation via the proteasomal pathway (FIG 17) [216]. To distinguish between these two possibilities, it would be interesting to investigate whether the fusion of heterologous E3 ubiquitin ligases to Vpr would also lead to the degradation of the proposed factor(s) and a G2 cell cycle arrest.

1.6.2. HIV-1 Nef regulates the levels of cell surface proteins

The HIV-1 negative factor, Nef, is a multifunctional 206 amino acid long protein (FIG 2) that is associated with the cytosolic site of plasma membranes via an N-terminal myristyl anchor. Nef is conserved across diverse primate lentiviruses including SIVs and HIV-2 and is expressed very shortly after infection in a Rev independent manner. Despite its name, Nef has positive effects on HIV-1 replication (FIG 17).

The first evidences that Nef modulates the pathogenicity of an infection *in vivo* came from experiments in which rhesus macaques were infected with a Nef deleted SIVmac239 [217]. The infected macaques showed higher CD4+ T-cell counts and none out of six SIVmacΔNef infected animals died, whereas three out of seven animals infected with wild type virus died. Sequencing HIV-1 isolates from blood transfusion recipients that had been infected but remained free of AIDS symptoms for up to 14 years, revealed deletions in the Nef coding region, demonstrating an impact of Nef on the progression of HIV-1 infected individuals towards AIDS [218].

An interesting ability of Nef is to down-modulate surface expression of diverse plasma membrane proteins including major histocompatibility complexes (MHC) class I and II, CCR5, CXCR4, CD4 and T-cell receptor (TCR-CD3) [219]. The down-modulation of surface levels of CD4 ensures efficient virus production, likely due to preventing interference of CD4 with gp120/ gp41 incorporation into budding virions [220, 221]. Nef binds the cytoplasmic tail of CD4 and recruits the clathrin adaptor protein complex 2 (AP-2), which leads to the formation of clathrin coated pits, internalisation and lysosomal degradation of CD4 [222].

Virus mediated decrease of MHC levels is a way to counteract the host immune system. In the presence of Nef, MHCs loaded with viral antigens are less efficiently presented at the cell surface, therefore cytotoxic T-lymphocytes (CTLs) are less efficiently activated ultimately benefiting the virus. However, since defined CTL escape mutations arise during HIV-1 infection in Gag, Tat and also Nef, the Nef impact on MHC presentation is not complete [223]. In contrast to downregulation of cell surface CD4 levels, Nef can decrease MHC levels by two different mechanisms: I) by interfering with their transport from the trans-Golgi network to the cell surface through recruitment of AP-1 complexes which direct them to endosomes [224] or II) by inducing a signalling cascade that ultimately leads to the endocytosis of cell surface MHC complexes [225].

Recent data suggests that Nef proteins from certain SIVs may also be able to counteract the antiviral protein tetherin, maybe through a similar mechanism as seen for CD4 cell surface downregulation (FIG 17) [194].

Nef also interacts with the cytoplasmic tail of the TCR-CD3 complex, decreasing the TCR-CD3 signalling in infected T-cells. This probably occurs via interaction with the CD3 ζ chain causing the inhibition of T-cell activation by blocking N-Wasp mediated Pak-2 signalling [226] as well as decreased clustering of the kinase Lck and TCR in immunological synapses [227]. HIV-1 Nef however does not change the overall levels of TCR-CD3 at the cell surface. This stands in contrast to Nef alleles from less pathogenic HIV-2 and most SIVs, suggesting that the disfunction of efficient TCR-CD3 downregulation by HIV-1 Nef may contribute to an increased pathogenicity [228].

Like Vpr, Nef is incorporated into virions [229]. Nef deleted virus is attenuated and this may depend on the route of infection of the target cell. VSV-G pseudotyped ΔNef virus is similarly infectious as wild type virus [230]. In contrast, ΔNef virus is attenuated when the virions harbour HIV-1 Env or amphotropic Env from MLV. Furthermore, the infectivity of ΔNef virus can be rescued when Nef is provided in *trans* in virus producer

cells, but not in target cells [231]. Recently, Vpr's characteristic of being packaged was exploited to analyse whether Nef incorporation is sufficient for rendering virus particles infectious [232]. The data showed that a fusion protein between Vpr and Nef with a central protease cleavage site was incorporated into virions and efficiently cleaved. However, resulting viruses were as attenuated as the ΔNef virus [232]. This suggests that Nef acts during the assembly of virions or that the generated Nef was not functional. Recent work also proposes that dynamin-2 and clathrin are involved in the effect of Nef to enhance infectivity of virions [198]. The authors show that Nef binds to dynamin-2 and enhances infectivity of released virions. Inhibition of dynamin-2 by dominant negative versions specifically inhibited the Nef effect in an Env dependent manner as described above.

1.6.3. HIV-1 Vif counteracts APOBEC3 proteins

The HIV-1 virion infectivity factor (Vif) is a 192 amino acid long protein that has been known for a long time to influence the infectivity of HIV-1. Deletion of the *vif* gene rendered HIV-1 non-infectious in some cells (e.g. HUT78, CEM), whereas no infectivity was lost in others (e.g. SupT1, CEM-SS). In a series of elegant experiments Sheehy and colleagues demonstrated that human APOBEC3G is necessary and sufficient to inhibit HIV-1 in the absence of the viral *vif* gene when expressed in permissive CEM-SS cells [196]. APOBEC3G (A3G) is a member of the apolipoprotein B mRNA-editing catalytic polypeptide 1-like protein 3 (APOBEC3) protein family. In addition to A3G, A3F can block Vif deficient virus and is also counteracted by the HIV-1 Vif protein in a similar way to A3G [233]. Furthermore, human A3B, rat A1 and murine A3 are able to inhibit HIV-1 but are insensitive to HIV-1 Vif [234]. Despite the fact that HIV-1 encodes a countermeasure, A3G may impact on the clinical progression of HIV-1 infection towards AIDS [235]. This may be due to different expression levels of A3G between individuals infected with HIV-1, or due to differential expression levels in cell subsets within an individual. Indeed, a recent study suggests that CD4+Th1 cells have higher levels of A3G than CD4+Th2 and virions derived from CD4+Th2 cells are more infectious [236]. CD4+Th1 cells develop from naive T-cells in an environment of high levels of interferon alpha (IFN-α) and themselves secrete interferon gamma (IFN-γ). Although it has been controversial, evidence that A3G is upregulated by both IFN-α and IFN-γ has recently increased [237].

A3G is packaged into virions in the absence of the viral Vif protein and causes its inhibitory effect on virus replication after the infection of a target cell (FIG 17) [238]. However, how A3G is packaged into the virions in the absence of Vif is unknown. Currently, Vif's function seems to be solely to inhibit members of the APOBEC3 family. A proposed mechanism of the antiviral activity of A3G, is the induction of deleterious hypermutations in the viral genome, a result of DNA cytidine-deamination on the minus strand during reverse transcription [238, 239]. A3G has two canonical cytidine deaminase domains (CDA), however only the C-terminal CDA mediates deamination [240]. In single stranded DNA, A3G deaminates cytidine (C) to uracil (U). Since the majority of single stranded viral DNA during reverse transcription is of negative polarity, C to U mutations result in guanosine (G) to adenosine (A) mutations in the plus strand. Uracil residues are excised by uracil DNA glycosylases and replaced with thymidine (T). In some positions on the viral genome like the PPT or the LTR regions, where plus stranded DNA is single stranded during reverse transcription, C to T mutations are also frequently observed [241].

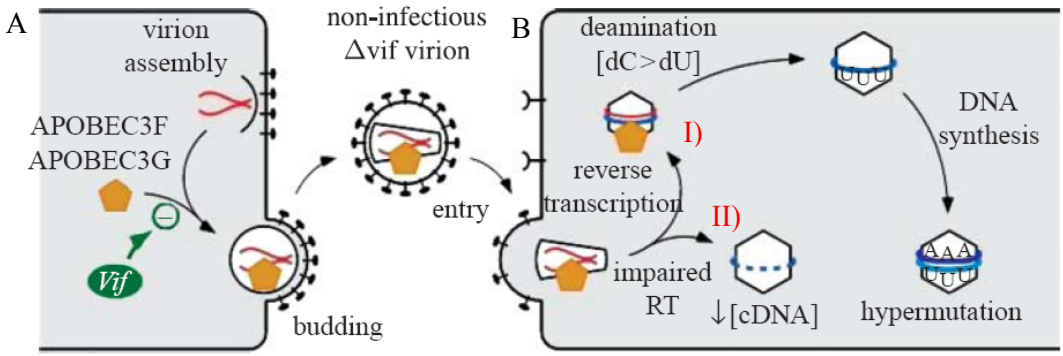


FIG 18 Antiviral activity of APOBEC3G/F in the retroviral life cycle

A) APOBEC3G and APOBEC3F (A3G/F) are counteracted by the HIV-1 Vif protein, preventing incorporation of A3G/F. Virus lacking Vif incorporates A3G/F which exerts its antiviral effect in the infected target cell by two mechanisms I) inducing hypermutations due to its cytidine deaminase of single stranded DNA and II) by interfering with elongation of reverse transcription, thereby reducing viral cDNA levels. Figure adapted from [242].

Although the editing activity of A3G has been suggested to play a role in restricting retroviral infection, recently it has become evident that A3G's and A3F's antiviral effects do not entirely depend on it. Newman et al. discovered that A3G mutants that have lost editing activity were still able to potently inhibit viral infectivity [243].

Moreover, a rescue of the A3G mediated reduction in cDNA could not be observed in cells with inhibited or absent uracil DNA glycosylases UNG2 or SMUG1, suggesting that these enzymes are not necessary for A3G's antiviral effect [244, 245]. A3G oligomerises in a RNA-dependent manner, suggesting that A3G might be packaged by interaction with the viral RNA [240]. A3G has been proposed to interfere with diverse steps during the process of reverse transcription, including tRNA annealing and priming of RT [246, 247], minus and plus strand transfer [248], and also provirus formation by interaction with IN [249] (FIG 7). A recent study demonstrated that an important mechanism for the hypermutation independent activity of A3G is likely to be its interference with reverse transcription elongation [195]. In this model A3G bound to viral RNA sterically hinders reverse transcriptase, inhibiting efficient cDNA synthesis. Using different primer/ probe sets in a quantitative PCR to detect viral cDNAs of different lengths, the study showed that the effect of A3G increases with increasing length of cDNA [195]. This suggests that A3G bound to viral RNA may interfere with cDNA elongation (FIG 18BII).

HIV-1 Vif prevents the incorporation of A3G into virions by recruitment of the cullin5-elonginB/C ubiquitin ligase complex [250]. The recruitment of an as far unknown E2 ligase via cullin5 consequently leads to ubiquitination of A3G and degradation by the proteasome so that produced virions are free of A3G [251, 252]. However, a recent study suggests that A3G ubiquitination is not necessary for a Vif-dependent degradation by the proteasome, suggesting that other factors in the complex, possibly Vif itself, may be a target for ubiquitination causing degradation of the whole complex [253]. The ability of Vif to counteract A3G is dependent on the phosphorylation status of serine residue 144 in the suppressor of cytokine signalling (SOCS)-box of Vif, which interacts with elongin C and thereby promotes cullin5 recruitment. Phosphorylation at this residue reduces its interaction with elongin C and consequently decreases degradation of the complex and anti-A3G activity [254]. Phosphorylation of Vif has been long known to influence its ability to enhance HIV-1 infectivity. Yang et al. demonstrated in 1996 that Vif is phosphorylated and showed two years later that the mitogen activated protein kinase (MAPK) phosphorylates Vif at residue S144 [255, 256]. Interestingly, a recent study showed that Vif interaction with A3G is strengthened by protein kinase A (PKA) mediated phosphorylation of A3G residue T32 [257]. Studies performed before the discovery of A3G showed, that Hck, a tyrosine kinase from the Src family, was able to inhibit ΔVif but not wild type HIV-1

and Vif was not the target of phosphorylation [258]. It would be interesting to investigate whether Hck is able to phosphorylate T32 in A3G.

APOBEC's sensitivity to Vif antagonism is species-specific. More precisely, human A3G cannot block wild type HIV-1, whereas A3G from e.g. African green monkeys (AGM) is able to block wild type HIV-1. Vice versa, SIVagm Vif is able to counteract AGM A3G, but not human A3G. Species-specificity is therefore a result of the inability of HIV-1 Vif to bind A3G of e.g. AGMs [259]. Interestingly, changing only one amino acid residue in human A3G to the residue found in AGM (D128K), disrupts interaction with HIV-1 Vif and confers the ability to bind SIVagm Vif, thereby switching species-specificity [260].

APOBEC proteins have been implicated as antiviral factors against other viruses including hepatitis B virus (HBV), adeno-associated virus (AAV), as well as against endogenous retrotranspositions of Alu segments. It is clear that the APOBEC family represents an important set of proteins to counter viral, in particular retroviral, infection and cross-species transmission.

1.6.4. HIV-1 Vpu counteracts tetherin/ BST2/ CD317

The HIV-1 unique viral protein Vpu is a ~16 kDa large type I transmembrane protein localised to the plasma membrane as well as intracellular membrane compartments. In the primate lentivirus lineage only HIV-1, SIVcpz and SIVgsn have Vpu proteins, whereas for example HIV-2 and SIVmac lack Vpu. Vpu is a viroporin and functions as a monovalent cation ion channel, although the importance of this function for the virus life cycle is not clear [261]. Vpu has an N-terminal transmembrane domain and a C-terminal cytoplasmic domain, which is a target for casein kinase II mediated phosphorylation [262]. Phosphorylation of the cytoplasmic domain is important for Vpu's ability to downregulate CD4 expression, whereas it is not involved in the positive effect of Vpu on virus release [263]. Like Nef (see 1.6.2.), Vpu can decrease surface levels of CD4 by mediating its degradation [264]. However, unlike Nef, which induces lysosomal degradation of CD4, Vpu mediated degradation involves the ubiquitin-conjugating and proteasomal pathway [265]. CD4 degradation is mediated via Vpu binding to the cytoplasmic tail of CD4 at the endoplasmic reticulum and the recruitment of two cellular factors, beta TrCP1 (and possibly TrCP2) and Skp1p, which form a

complex that initiates ubiquitination and degradation [266, 267]. However, the specific target for ubiquitination remains unknown.

Augmentation of HIV-1 release in the presence of Vpu, and impaired virus release and accumulation of cell associated viral proteins in its absence, was observed early after identification of HIV-1 [268]. Moreover, it was soon realised that the positive effect is transferable to other retroviruses, suggesting that a cellular factor non-specifically repressed virus release in a retrovirus common pathway [269]. It was also shown that the protein targeted by Vpu, resided in the plasma membrane and prevented the release of nascent particles [270]. In addition, the effect of Vpu could be overcome by pretreating infected cells with interferon [271]. Together these observations led to the discovery of the antiviral activity of a protein known as bone marrow stromal factor 2 (BST2) or CD317 [193, 272]. Due to its ability to inhibit HIV-1 release by tethering nascent virions to the plasma membrane, it is now also referred to as tetherin (FIG 17). Tetherin is a transmembrane protein with an N-terminal cytoplasmic domain, followed by a transmembrane helix, an extracellular alpha helical domain and a C-terminal glycosylphosphatidylinositol (GPI) anchor (FIG 19). It is heavily glycosylated at amino acid residues N-terminal of the extracellular alpha helical domain. Recent data suggests that N-terminal glycosylation may be dispensable for tetherin mediated restriction of virus release [273].

Vpu may mediate tetherin degradation in a proteasome dependent manner [274, 275]. This was suggested to be cell type specific, since increased virus release by Vpu was observed without a decrease of cell associated tetherin in some cell lines, e.g. H9 [276]. However, since HIV-1 infectivity in these cells was only rescued by maximal 3-fold in the presence of Vpu, it is very likely that the loss of cell associated tetherin was below detectable levels. It is possible that Vpu causes tetherin degradation by recruitment of the beta TrCP1/Skp1p complex, followed by ubiquitination and proteasomal degradation, similarly to its effect on CD4. In addition, a recent study suggests the involvement of the endolysosomal pathway for Vpu mediated degradation of tetherin [277].

HIV-2 does not possess a Vpu protein but the HIV-2 envelope protein has been demonstrated to have Vpu like activity and to enhance viral budding [278, 279]. Indeed, a recent study suggests that HIV-2 Env is able to counteract tetherin [194]. Human tetherin also restricts MLV, however it is currently unknown whether murine tetherin restricts MLV and if so, how MLV counteracts tetherin restriction. In addition, the

unrelated Ebola virus is restricted by tetherin and counteracts the block with its envelope glycoprotein [271, 280]. For most viruses restricted by tetherin, the viral countermeasures remain unidentified. However, it was demonstrated that human tetherin restricts VSV-G pseudotyped VLP production of EIAV, FIV, SIVmac, SIVagmSab, Rous sarcoma virus (RSV), Mason Pfizer monkey virus (MPMV), a reconstituted human endogenous retrovirus (HERV-Kcon), human T-cell leukaemia virus (HTLV-I), as well as primate foamy virus (PFV) [281].

Tetherin has been positively selected during primate evolution and acts in a species-specific way, e.g. Tantalus monkey tetherin is able to block HIV-1 virus release however is not counteracted by HIV-1 Vpu [274, 282]. Moreover, mouse, rat and rhesus macaque tetherins efficiently block HIV-1 and Vpu is also unable to rescue release [275, 282]. Positively selected tetherin residues are located close to or within tetherin's transmembrane helix. A recent study suggests that tetherin residues L22 to I46 constitute the alpha-helical transmembrane domain and that helix residues P40 and T45 are critical for the sensitivity to HIV-1 Vpu [282]. Mutation of human tetherin residues P40 or T45 to residues found in Tantalus monkey tetherin, render the human protein insensitive for counteraction by HIV-1 Vpu [274, 282]. So far, the Vpu determinants for species-specificity have not been mapped. However, Vpu's transmembrane region seems to be important. It would be interesting to compare Vpu proteins from closely related SIVcpz and HIV-1, which might reveal determinants of Vpu for tetherin counteraction. Most SIVs, including SIVmac, do not possess a Vpu protein. Recent data suggests that they counteract tetherin restriction with their Nef protein providing yet another way to escape innate immunity [194].

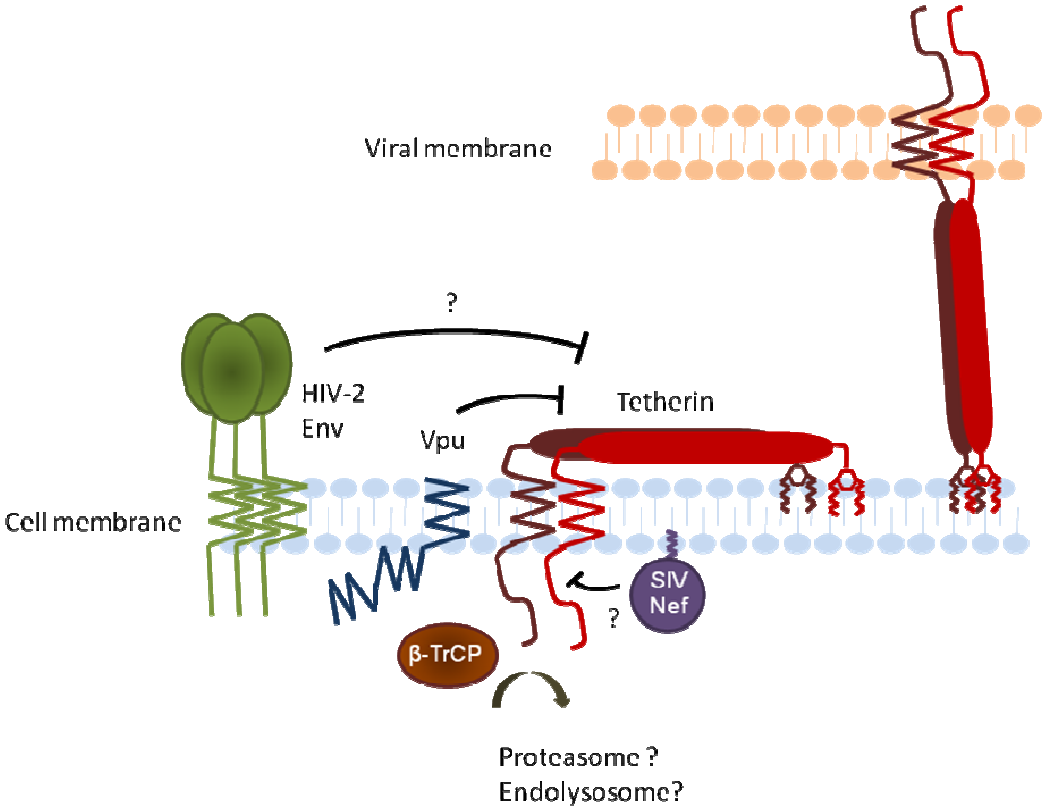


FIG 19 Tetherin restricts virus particle release and is counteracted by Vpu, HIV-2 Env and SIV Nef

Tetherin functions to tether nascent virus particles to the plasma membrane causing their endosomal take up and degradation. Vpu is able to counteract tetherin possibly by β-TrCP mediated endolysosomal or proteasomal degradation. HIV-2 Env counteracts tetherin by an unknown mechanism. Nef proteins from SIVs may also counteract tetherin, however the mechanism is not known.

1.7. Fv1 restriction of MLV

A subgroup of intrinsic factors includes molecules that act shortly after the retroviral capsid has entered the cytoplasm of a target cell. The mouse Friend virus susceptibility 1 (Fv1) gene encodes the prototypic restriction factor. It is an almost full-length endogenous retroviral Gag protein encoded by mice [188]. It is one of the genes controlling the susceptibility of mice to infection by murine leukaemia virus, originally described in the early 1970's [283, 284]. Interestingly, Fv1 homologues are only found in mice, but not in rats, suggesting that the virus from which Fv1 derives infected mice after the divergence of *Mus* and *Rattus*. In an extensive study Best and colleagues were able to identify two major alleles from AKR and C57BL/6 mice, Fv1N and Fv1B, respectively [188]. N tropic MLV (MLV-N) is restricted by Fv1B but not Fv1N, therefore infects AKR but not C57BL/6 or BALB/3T3 mice. MLV-B has the opposite phenotype. MLV-N and MLV-B differ in several amino acids, however switching CA amino acid residue 110 is sufficient to switch the tropism to Fv1 restriction [285]. MLV-N with R110 substituted to the residue found in MLV-B (E110) loses sensitivity to Fv1B and gains sensitivity to Fv1N restriction [285]. In addition to a longer C-terminus, Fv1B differs from Fv1N in two internal positions 358 (E/K, Fv1B/Fv1N) and 399 (R/V, Fv1B/Fv1N) and all three differences are important for specificity for MLV-N or MLV-B restriction (FIG 20) [188, 286]. In addition, other alleles of Fv1 have been described. Naturally occurring MLV-N like viruses have been found that escape restriction by these alleles without changing CA residue R110 but due to changes at other CA positions suggesting that CA residue 110 is not solely determining tropism to Fv1 alleles [287]. MLV restricted by Fv1 completes viral DNA synthesis by reverse transcription but does not form a provirus (FIG 17) [288, 289]. In addition, viral DNA circles are reduced for Fv1 restricted MLV, suggesting that the block occurs before nuclear entry of PICs (FIG 17) [288, 289]. A deletion study by Bishop et al. clearly showed that the C- and N-terminus of Fv1 are important for restriction of MLV, whereas internal deletions had only minor effects on the restriction ability [290]. The crystal structure of Fv1 has not been published yet. However, biochemical investigation of Fv1 suggested that it consists of an N-terminal domain (residues K16 to R157), a central domain (residues 158 to 243) and a C-terminal domain (S244 to L440) (FIG 20) [291]. The N-terminal domain has a predicted coiled coil between residue 88 and 115, therefore possibly acts as a dimerisation domain,

whereas the carboxy-terminus presumably functions as the virus recognition domain and interacts with the MLV capsid [291]. The C-terminus also contains the major homology region of retroviral Gag proteins between residue 267 and 286 [292]. Mutation of the MHR abrogates restriction [290]. The details of the molecular mechanism underlying restriction by Fv1 are unclear, but current data suggest that retroviral capsids interact early after entry with Fv1 rendering them non-infectious [293].

Interestingly, a block to viral infection in certain primate and human cell lines to MLV infection was described, which was similar to the Fv1 phenotype in murine cells, despite the lack of an *Fv1*-like gene in those cell lines [294, 295]. Experimental analysis of that observation led to the discovery of the alpha isoform of the cytoplasmic body component tripartite motif protein 5 (TRIM5α) as a potent antiretroviral protein (see 1.8.) [184, 296-298]. TRIM5α blocks viral infection early after virus entry and in most cases, unlike Fv1, by inhibition of viral DNA synthesis (FIG 17) [184]. When TRIM5α and Fv1 are expressed together, both restriction factors compete for binding to incoming viral cores however the actual restriction mechanism occurs late for Fv1 and early for TRIM5α, as judged by levels of viral DNA reverse transcription products [293]. Despite the differences in the stage of the block to viral infection, some aspects of MLV restriction by Fv1 are similar to restriction by TRIM5α. Like TRIM5α, Fv1 is saturable and both factors target the incoming viral CA protein. The block of MLV-N by both, Fv1N and human TRIM5α, depends on R110 in the MLV CA protein [285, 294, 299, 300]. In addition, both molecules are believed to form cytoplasmic dimers consisting of a C-terminal virus binding moiety and an N-terminal domain, which may be involved in dimerisation [291, 301]. Like TRIM5α, Fv1 seems to be positively selected, suggesting that it is under selection pressure from MLVs in mice [302-305].

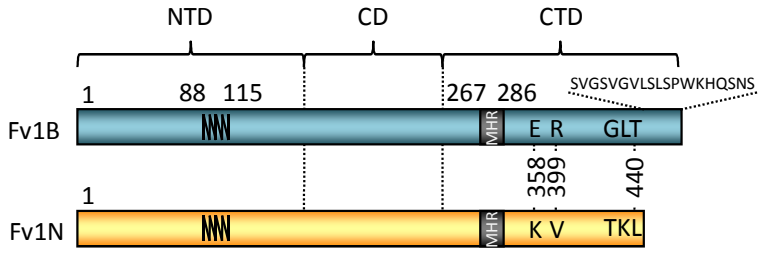


FIG 20 Schematic protein organisation of Fv1N and Fv1B

Figure shows Fv1B (top) and Fv1N (bottom) domain organisation. NTD, N-terminal domain; CD, central domain; CTD, C-terminal domain; MHR, major homology region. Predicted N-terminal alpha helical domain is indicated.

1.8. TRIM5α

TRIM5α was first isolated in 2004 from a rhesus macaque cell line and shown to block HIV-1 infection of old world monkey cells [184].

As a typical TRIM protein, the alpha splice form of TRIM5 is comprised of a RING, B-box, coiled-coil domain and a C-terminal B30.2 or PRY-SPRY domain. The latter harbours the major virus recognition determinants [306-309], with which dimeric TRIM5α complexes bind to incoming retroviral capsids. An early study on the oligomerisation proposed that TRIM5α oligomerises as homotrimers [310]. However, more recent *in vitro* biochemical assays with recombinant TRIM5α suggest that it forms homodimers and that dimeric B30.2 structures bind viral CA structures [301]. TRIM5 is alternatively spliced into different isoforms of which only the alpha isoform has an antiviral activity due to its virus binding B30.2 domain. Shorter TRIM5 isoforms β, γ, δ and ε have been described (FIG 21) [311]. Isoforms δ and γ, which both lack the B30.2 domain, have been shown to interfere with restriction presumably due to the disruption of homodimeric TRIM5α complexes or saturation of co-factors [184, 293]. Like many other TRIM proteins, TRIM5α is interferon inducible supporting its important function as a restriction factor [312-314]. In contrast to restriction by Fv1, most TRIM5α proteins block virus infection before reverse transcription is complete (FIG 17) [184]. However, the block to RT can be largely abrogated by chemical inhibition of the proteasome [315]. Formation of 2-long terminal repeat (2-LTR) circles and infection cannot be rescued by proteasome inhibition [316]. The role of the RING, B-box 2 and coiled-coil domains in the antiviral function of TRIM5α remain largely unclear but mutation studies indicate that all three domains are important for full antiviral function of TRIM5α [317-321]. In addition to an early block after virus entry, rhesus macaque TRIM5α has been proposed to interfere with HIV-1 particle production (FIG 17). However, this role remains controversial and contradicting results have been published [201, 202].

TRIM5α belongs to the large family of TRIM proteins with diverse functions. Despite many functional differences TRIM proteins have common features. The most obvious shared characteristic between TRIM proteins is the RBCC or tripartite motif consisting of three characteristic domains, the RING, the B-box and the coiled coil domain. The order of the three domains and the spacing between the individual domains is highly conserved between TRIM family members.

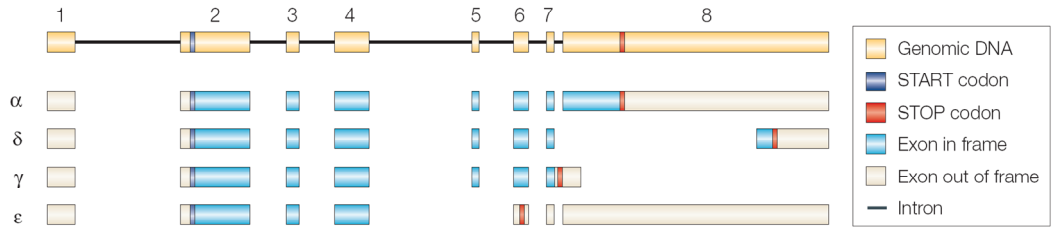


FIG 21 TRIM5 splice isoforms

The human TRIM5 gene is shown and the different splice isoforms TRIM5α, δ, γ and ε are indicated. Figure adapted from [322].

1.8.1. The RING domain

The really interesting new gene (RING) domain is common to TRIM proteins and is the most amino-terminal domain of the tripartite motif. The length and the start of the RING domain after the methionine start codon varies between TRIM proteins [323]. The RING domain is a zinc finger motif defined by the consensus sequence $\mathbf{C}X_2\mathbf{C}X_{(9\text{-}39)}\mathbf{C}X_{(1\text{-}3)}\mathbf{H}X_{(2\text{-}3)}\mathbf{C}/\mathbf{H}X_2\mathbf{C}X_{(4\text{-}48)}\mathbf{C}X_2\mathbf{C}$ in which the histidine (H) and cysteine (C) residues indicated in red coordinate in total two zinc ions [324]. The structure of the human TRIM5 RING domain has been solved and is depicted in FIG 22A. Initially, the RING domain was shown to possess a E3 ubiquitin-ligase activity in TRIM5δ, a C-terminally truncated isoform that associates with the topoisomerase-I interacting proteins BTBD1 and BTBD2 [325]. A recent study showed that also human TRIM5α possesses an E3 ubiquitin-ligase activity and in addition to self-ubiquitination can also be ubiquitinated by TRIM21 *in vitro* [326]. The function of the RING domain in TRIM5α restriction is unclear and is a subject of this study (see 5.2.).

RING domains of many TRIM family members including TRIM5, TRIM11, TRIM21, TRIM22 and TRIM25 have been demonstrated to have E3 ubiquitin-ligase activity [326-330]. In addition to E3 ubiquitin ligase activity some TRIMs may also have an E3 small ubiquitin-related modifier (SUMO) ligase activity. In particular, TRIM19 and TRIM63 have been described to bind to SUMO-conjugating E2 enzyme Ubc9 [331, 332]. In addition, TRIM27 interacts with the SUMO conjugating ligase PIAS [333]. TRIM25, also known as estrogen-responsive finger protein (EFP), is able to mediate the transfer of yet another ubiquitin-like protein called ISG-15 [334]. Mutation of the

TRIM25 RING domain led to a diminished ISGylation of its target protein 14-3-3σ indicating the importance of the RING domain in this reaction [334].

Evidence for functional importance of the RING domain in TRIM proteins are provided by the observations that RING deletion mutants may have aberrant subcellular localisations compared to wild type proteins, as seen for example for TRIM6 and TRIM8 [311]. In addition, mutation of the cysteines involved in zinc-coordination often abolishes TRIM protein function. This was, for example, observed for murine TRIM30 mediated degradation of TAB2 [335]. Mutation of cysteine C15A in human or rhesus macaque TRIM5α resulted in the loss of self-ubiquitination and significantly decreased antiviral activities [184, 326].

However, the function of some TRIM proteins does not entirely depend on an intact RING domain. A deletion study on TRIM27 for example demonstrated that the RING and B-box domains are dispensable for the effect on inhibiting NF-κB activity and IRF3 nuclear translocation [336]. Moreover, some TRIM family members do not possess a RING domain however their remaining domain organisation resembles that of other TRIM proteins justifying a classification within the family. For example TRIM14, TRIM16, TRIM20, TRIM29, TRIM44 and TRIM66 lack a RING domain however all of them contain at least one B-box domain [311, 322].

1.8.2. The B-box domain

B-box domains are found in almost all TRIM family members. Two different types of B-box domains, B-box1 and B-box2, can be distinguished according to their consensus sequences. Interestingly, a B-box2 domain will always follow a B-box1 domain, and only B-box2 domains are found alone, suggesting that B-box1 domains may have evolved from B-box2 domains. Using bioinformatic and biochemical approaches, a recent study suggests an importance of the ~30-48 amino acid residues long region located between the RING domain and the first B-box domain, which has been misleadingly called RING-B-box-Linker (RBL) region [337]. The study identified this region to be unique to TRIM proteins and showed that both, the RING domain and the RBL alpha helix are mutually dependent on each other with regards to zinc-coordination and helix formation. This strengthens the notion that the RBCC motif is conserved in its domain organisation and spacing, and that the domains act as a single unit. Like RING domains, B-box domains also coordinate zinc and have a similar ternary structure to

RING domains, suggesting that they may share similar functions and may have evolved from a common ancestral domain [338]. Information on the function of B-box domains are limited and mainly come from deletion or mutation studies. Deletion of the B-box2 domain in TRIM6 or TRIM29, for example led to an aberrant subcellular localisation compared to the wild type proteins and to a loss of self-association, in case of TRIM29 [311]. However, these observations are likely to be artefacts of perturbed protein folding.

The structure of the human TRIM5 B-box domain is depicted in FIG 22B. The B-box domain is a B-box of type 2, consists of three antiparallel β-sheets and, like the TRIM5 RING domain, coordinates two zinc ions. The function of the B-box domain of TRIM5 is unclear however a similar structure to the RING domain may suggest redundant functions. Moreover, an intact B-box is important for TRIM5α's antiviral activity. The function of the TRIM5α B-box domain in the restriction mechanism is the subject of this study (see 5.2.1.).

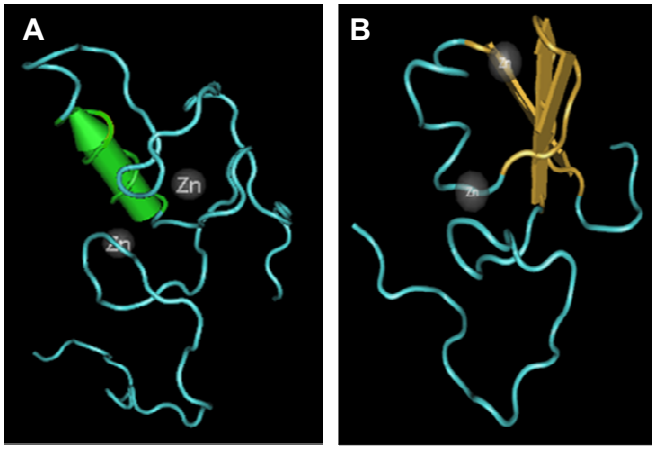


FIG 22 Structures of the human TRIM5 RING and B-box domains.

RING domain residues M1-E78 (A) and B-box residues S86-F129 (B) are shown. RING α-helix from Q36-K45 is shown as a green tube. Antiparallel β-sheets between L103-I113 and H126-F129 in the B-box are shown as brown arrows. (Entrez structure PDB IDs: RING, 2ECV; B-box, 2YRG; pictures derived using Cn3D4.1)

1.8.3. The coiled coil domain

The coiled coil domain of TRIM proteins is important for oligomerisation of monomers, which in case of TRIM5α most likely results in dimers [301, 339]. Interestingly, despite the large number of TRIM family members the majority of TRIM proteins form

homomultimers and only a few have been described to form heteromultimers, including TRIM1, TRIM18, TRIM19, TRIM24, TRIM27, TRIM29 and TRIM31 [311]. TRIM proteins have defined subcellular localisations and the correct localisation depends on the coiled coil domain since deletion of the domain often results in a diffuse distribution of the protein within the cell [311]. The coiled coil domain is found in almost every TRIM protein. However, some members, in particular TRIM48, TRIM49, TRIM52, TRIM53 and TRIM61 have no predicted coiled coil structures, suggesting that they may act as monomers [340].

Analysis of the secondary structure of the TRIM5α coiled coil domain by computer based prediction suggested that two separate regions, which both form coiled coils, are linked by a spacer region. Mutational analysis of the three regions showed that all three regions contribute to oligomerisation of TRIM5α molecules, however mutations in the more carboxy-terminal coiled coil were more deleterious to the ability of the protein to restrict than mutations in the amino-terminal part [341].

In contrast to owl monkey TRIMCyp, the rhesus TRIM5 coiled coil domain could be substituted by coiled coil domains of other TRIMs without significantly affecting the antiviral activity [341]. This result seems rather surprising given the fact that many TRIMs have been described to be able to restrict HIV-1 when fused to the owl monkey TRIMCyp CypA domain [342]. It suggests different functional requirements for the coiled coil domain between TRIMCyp and rhesus TRIM5α. In fact, HIV-1 infection restricted by TRIMCyp was more readily rescued by expression of coiled coil domains from e.g. TRIM27 than HIV-1 restricted by rhesus TRIM5α [341]. In addition, expression of the owl monkey TRIMCyp coiled coil domain rescued infection of HIV-1 in owl monkey kidney cells [343]. Expression of the rhesus TRIM5α coiled coil domain in rhesus TRIM5α expressing cells also rescued infectivity of HIV-1 [341]. Yeast-two hybrid assays also showed that the human TRIM5α coiled coil domain alone is able to bind to full-length human TRIM5α [344]. In conclusion, the TRIM5α coiled coil domain is important for dimerisation of TRIM5α and for its function as a restriction factor.

1.8.4. The B30.2 domain

Domains that are located C-terminal to the tripartite motif differ dramatically in their sequences between different TRIM proteins (FIG 24). Although some TRIM proteins remain unclassified, most family members have been grouped into 11 sub-families

according to their domain organisation [323, 340]. The biggest class (C-IV) contains members that possess a PRY-SPRY motif. In many cases it is thought that the C-terminal domain acts as a binding domain, which recruits the RBCC motif to exert its enzymatic function on a certain protein that is targeted by the C-terminal domain. TRIM5α also possesses a B30.2 or PRY-SPRY domain, which was shown to bind to retroviral capsids (FIG 24).

In addition to B30.2 domains, further C-terminal domains that can be found in TRIM proteins include fibronectin type III motifs, **p**lant **h**omeo**d**omains (PHD), BROMO domains, transmembrane domains, filamin-type immunoglobulin domains, NHL (first characterised in proteins **N**CL-1, **H**T2A and **L**in-41) repeat motifs, **m**eprin **a**nd **T**RAF **h**omology (MATH) domains and **A**DP-**r**ibosylation **f**actor (ARF) domains. The C-terminus can provide clues for the function of the TRIM protein. For example TRIM28 possesses a BROMO domain, which is known to interact specifically with acetylated lysines. BROMO domain containing proteins are associated with chromatin and histone acetyltransferases. Indeed, TRIM28 was recently found to mediate provirus silencing of retroviruses in embryonic stem cells by binding to ZFP809, a protein that associates with the primer binding site of integrated retroviral DNA (FIG 17) [190, 345]. The importance of the enzymatic activity of the TRIM28 RBCC in this process has to be investigated.

The structure of the TRIM5α B30.2 domain has not yet been solved. Recently however, the PRY-SPRY structures of the *Drosophila melanogaster* protein GUSTAVUS and human TRIM21 were solved and show important features of the PRY-SPRY domain (FIG 23) [346, 347]. The B30.2 domain consists of beta sheets building the core and variable loops which extrude and form the surface of the domain (FIG 23).

The TRIM5α B30.2 domain harbours the determinants for recognition of the viral capsid (FIG 23) [184]. Not surprisingly, TRIM5α proteins from different species vary mostly in their B30.2 domains, in particular in four variable regions (v1-v4) (FIG 35), which differ in sequence and length, strongly suggesting that these represent the regions of interaction with viral capsids [348]. Using chimaeric proteins between human and rhesus TRIM5α, it was shown that v1 and v3 are important for MLV-N restriction by human TRIM5α [306]. In a similar but more complex study it was demonstrated that v1, v2 and v3 contribute to the binding specificity of the B30.2 domains of primate TRIM5α molecules to different viruses [308]. Changing 9 amino acid residues in the human TRIM5α B30.2 v1 region to the corresponding residues found in rhesus TRIM5α

allows this chimaeric protein to restrict HIV-1 potently by up to 10-fold [307]. Interestingly, changing a single arginine residue (R332) in the human TRIM5α B30.2 v1 region to proline found in rhesus TRIM5α (P332) has been shown to increase restriction of HIV-1, however the antiviral activity is weaker than that of the 9 amino acid substitution mutant [349]. A single amino acid change cannot only change but also can expand TRIM5α restriction specificity to other viruses. This has been demonstrated by mutating human TRIM5α B30.2 residue Y336, expanding its restriction to MLV-B and NB-tropic MLV [350]. Comparing Old World and New World primate B30.2 domain sequences, large differences in the variable regions can be detected. TRIM5α proteins from different African green monkeys (agm) have a 20 amino acid long duplicated sequence in v1, whereas the spider monkey TRIM5α shows a triplicated sequence in its v3 region suggesting a tremendous evolutionary pressure on these domains [348]. In addition, it was shown that the duplication found in agm TRIM5α is important for the restriction specificity of this protein [351].

Throughout primate evolution the B30.2 domain has changed significantly at these specific sites that interact with retroviral capsids, supporting the theory of co-evolution of host and retroviruses. This strong positive selection led to species-specific TRIM5α molecules restricting different retroviruses [303, 352, 353]. The virus that is found naturally in a host has escaped restriction by changing its CA sequence in a way that the host TRIM5α cannot recognise it. Rhesus TRIM5α for example is able to strongly block infection by HIV-1, whereas its human homologue only inhibits HIV-1 weakly [184]. In addition to interspecies variation, TRIM5α from rhesus macaques is also under strong selection within the species [352]. In contrast to human TRIM5α, multiple polymorphisms have been found in the rhesus macaque TRIM5α B30.2 domain, which affect restriction specificity of diverse retroviruses [352, 354, 355].

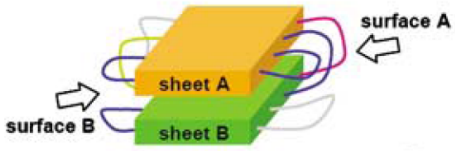


FIG 23 Structural organisation of the PRY-SPRY domain

A simplified cartoon structure is shown. The core of the domain consists of two sheets (sheet A and B), which are built of anti- parallel beta sheets (green and orange). Variable loops extrude and build different surfaces that can interact with target proteins. In TRIM5α surface exposed variable loops contain the species-specificity determinants for TRIM5α restriction and interact with retroviral capsids. Figure derived from [347].

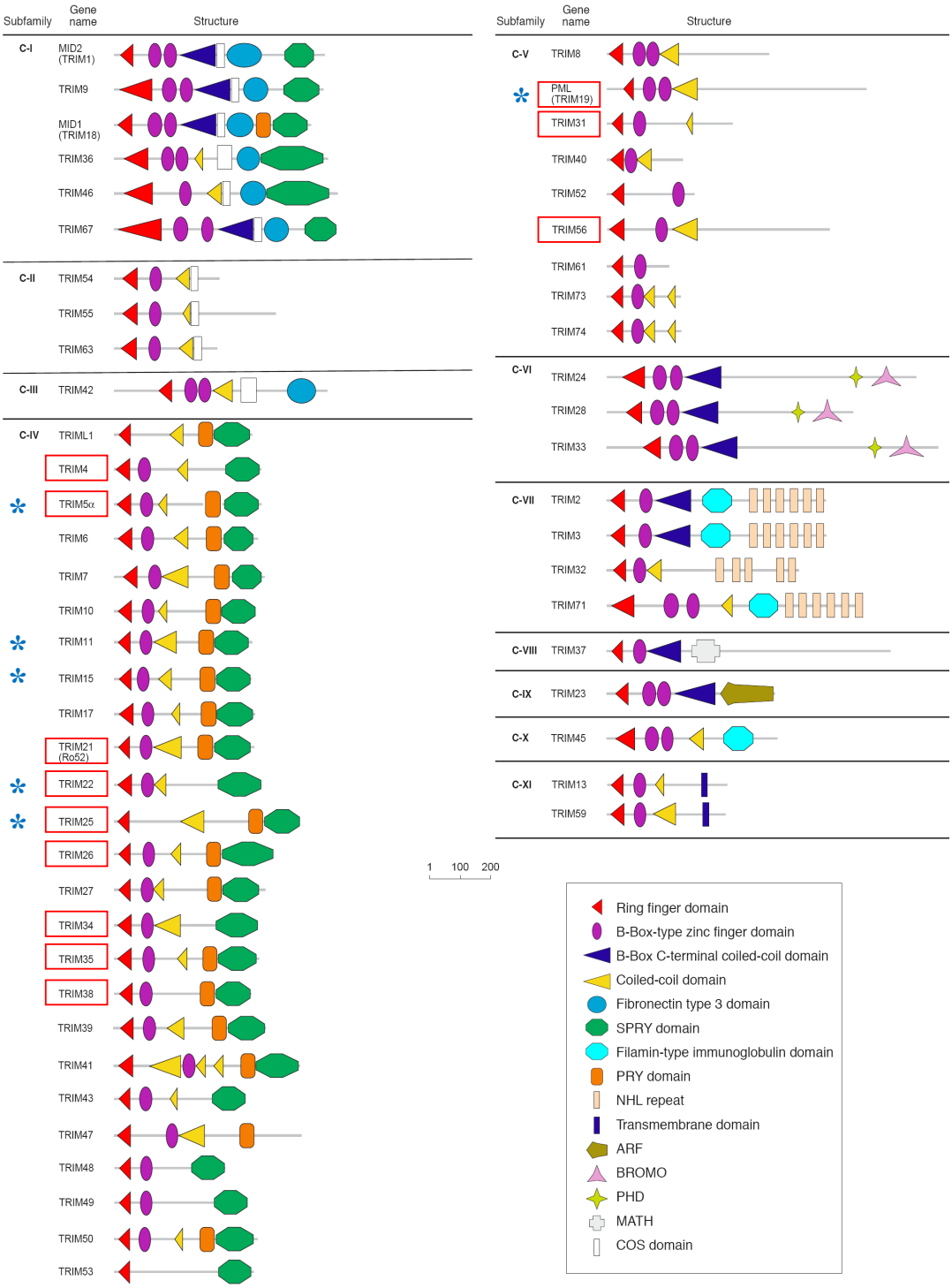


FIG 24 Sub-families of TRIM proteins

Tripartite motif (TRIM) proteins are grouped into 11 classes C-I to C-XI and some unclassified members (not depicted). The biggest class contains members that possess a PRY-SPRY C-terminal domain (C-IV). Proteins known to be involved in the antiviral response are indicated with an asterisk. TRIM proteins known to be upregulated by interferon are highlighted in red boxes. Most proteins known to be involved in antiviral or immune modulatory processes are found in C-IV. Scale is amino acid numbers. Figure adapted from [323].

1.8.5. Characteristics of TRIM5 expression

TRIM5α forms discrete concentrations in the cytoplasm, referred to as cytoplasmic bodies [184]. These may in some cases be aggresomes, which are thought to immobilise intracellular protein accumulations, rendering them susceptible to proteasomal degradation [356]. However, these subcellular structures have only been observed for exogenously expressed, tagged TRIM5α proteins and it was shown that pre-existing cytoplasmic bodies are not essential for TRIM5α restriction [357]. It is not clear whether endogenously expressed TRIM5α also forms cytoplasmic bodies since available TRIM5α antibodies are inefficient at detecting endogenous TRIM5α. The formation of cytoplasmic bodies likely depends on the concentration of the protein within the cytoplasm, which is also increased by stimulation with interferons [312, 358]. In addition to cytoplasmic bodies, TRIM5α is also found diffusely in the cytoplasm and it has been demonstrated that TRIM5α exchanges between cytoplasmic bodies and the diffuse pool constantly [359]. Interestingly, it has been shown that HIV-1 associates with cytoplasmic TRIM5α bodies after infection and that proteasome inhibition leads to the aggregation of HIV-1 viral complexes in these bodies and no loss of detectable viral capsids [360]. Aggresomes form also as an effect of autophagy and virus replication has been associated with autophagy induced aggresomes called autophagolysosomes [361]. In addition, autophagy induced aggresomes have been suggested to be involved in protecting cells from virus infection by mediating MHC class II presentation of viral antigens [362]. However, formation of TRIM5α aggresomes and restriction of virus infection by TRIM5α have not yet been linked to autophagy. It is possible however, that the block to infection after proteasome inhibition is caused by protein aggregation and autophagy of accumulated virus-restriction factor complexes. It might therefore be also an interesting hypothesis that restriction by TRIM5α may enhance MHC class II antigen presentation but this has yet to be investigated.

Microarray expression analyses comparing different tissues (GNF Expression Atlas 2 Data from U133A and GNF1H Chips; http://genome.ucsc.edu) demonstrated that human TRIM5α is proportionally more expressed in peripheral blood lymphoid cells like dendritic cells, monocytes, natural killer cells, CD8+ T-cells and CD19+ B-cells suggesting that it may have a protective role against retroviral infection of immune cells. However, since expression of TRIM5α is IFN inducible the array data may not reflect the situation after virus infection.

1.8.6. Other TRIM family members and their roles in innate immunity

The TRIM protein family is heterogenous and large, comprising 65 and 64 members in humans and mice, respectively (FIG 24) [322, 323]. Interestingly, worms and flies possess only ~20 and ~10 members, which indicates how the family has expanded during evolution [323]. Members of the TRIM family obtain diverse functions in a wide range of biological processes and many of them are associated with pathways of innate immunity often against viral infections. TRIM19 (also known as promyelocytic leukaemia protein, PML) for example is able to block herpes simplex virus (HSV) infection [363], may interfere with Adenovirus [364] and cytomegalovirus infection [365], may be targeted by the Epstein-Barr virus nuclear antigen 1 (EBNA1) protein [366] and has been suggested to mediate antiviral mechanisms against RNA viruses like poliovirus [367], Ebola virus [368], Lassa virus [369], Rabies virus [370] and influenza [371]. Furthermore, it has been proposed to exert an antiviral activity against HIV-1 at a step prior to nuclear translocation of the pre-integration complex [372], as well as against human foamy virus (HFV) by sequestering the transcriptional transactivator protein Tas and repressing transcription [373]. However, the biological relevance of the results obtained from most of these studies remains unclear.

Murine leukaemia virus (MLV) replication is restricted in embryonic stem cells by TRIM28 which mediates transcriptional silencing of the provirus by binding to the MLV primer-binding site (PBS) [190]. TRIM22 has been suggested to have antiretroviral activity and was shown to decrease HIV-1 production by approximately 4-fold in a human osteogenic sarcoma (HOS) cell line expressing CD4 and CXCR-4 [200, 374]. By using siRNA mediated knock down and exogenous expression experiments a recent study investigated a large group of TRIM family members for antiviral activities and showed that human TRIM11 and TRIM15 can inhibit HIV-1 and MLV virus release by 4- and 5-fold, respectively [199]. However, since these effects are small, the physiological relevance remains controversial.

In addition to a direct effect on virus replication TRIM family members can exert their effect indirectly through the regulation of interferon (IFN) induced signalling pathways or pathogen recognition pathways involved in the activation of nuclear factor κB (NF-κB) leading to the synthesis of IFN and other cytokines. Type-I IFNs (IFN-α and IFN-β) are produced by many cell types in response to viral infections. In contrast, type-II IFN (IFN-γ) is only synthesised by T-cells and natural killer cells. The released IFNs act to induce an antiviral state in cells by binding to its receptors and inducing

signalling cascades that increase the expression of many antiviral proteins. Expression of many TRIM proteins is changed in response to IFNs, suggesting that they may be involved in antiviral processes. TRIM family members that are upregulated in response to IFN include TRIM5 [312-314], TRIM8 [375], TRIM19 [376], TRIM21 [377], TRIM22 [378] and TRIM25 [314, 334]. Moreover, a recent study showed that gene expression of 27 of the 72 human TRIM proteins is sensitive to IFN type I or II treatment in primary blood lymphocytes or monocyte derived macrophages [340]. The upregulated TRIM proteins are shown in FIG 24. In addition, this study revealed that some TRIM proteins, in particular TRIM9 and TRIM54, are specifically upregulated in FcγR-activated macrophages, suggesting a role in the immune control of antibody opsonised pathogens. A study in mice showed that TRIM proteins are differentially expressed in macrophages, myeloid and plasmacytoid dendritic cells and in a $CD4^+$ T-cell subset in response to IFN suggesting that the function of TRIM proteins in innate immune control may vary in different immune cells [358].

TRIM25 acts in concert with the retinoic-acid-inducible gene I (RIG-I) protein that recognises viral RNAs [379]. By binding to RIG-I's first caspase-recruitment domain (CARD) via the PRY-SPRY domain, TRIM25 is able to ubiquitinate the second RIG-I CARD domain, which ultimately results in increased downstream signalling of RIG-I and IFN-β production and supports antiviral activity against RNA viruses [327, 380]. However, it is not known how RNA binding of RIG-I triggers TRIM25 activation.

Mouse TRIM30, the closest relative to TRIM5α proteins of other species, is able to bind to transforming growth factor-β-activated kinase 1 (TAK1) thereby initiating the degradation of TAK-1-binding protein 2 (TAB2) and TAB3, two proteins that are responsible for the activation of Iκ-B kinase (IKK) after Toll-like receptor (TLR) signalling, ultimately resulting in the inhibition of NF-κB activity [335]. In this way TRIM30 presumably regulates TLR signalling and inflammation. Similarly, human TRIM27 has the ability to bind to IKKs and thereby negatively regulates NF-κB and interferon response factor 3 (IRF3) activity [336].

In addition to the regulation of interferon and cytokine production by regulating pathogen recognition pathways TRIM proteins can also influence type-I and type-II IFN induced signalling pathways. IFN induced signalling pathways need to be tightly regulated to avoid uncontrolled inflammation. The suppressor of cytokine signalling 1 (SOCS-1) inhibits nuclear translocation of signal transducer and activator of transcription 1 (STAT-1) homodimers, which mediate IFN-γ signalling, and is itself

under negative regulation by TRIM8 [375]. Both, TRIM8 and SOCS-1 expression are stimulated by IFN-γ suggesting a mechanism for fine tuning of the IFN induced response.

TRIM21 may also be involved in responses to infections due to its ability to ubiquitinate IRF8, leading to an enhanced activity of this transcription factor and an increased cytokine production [381]. Indeed, when overexpressed in CD28-stimulated Jurkat T-cells, TRIM21 increases interleukin 2 (IL-2) production, and RNAi mediated reduction of TRIM21 reduces IL-2 synthesis [382]. TRIM21 was also shown to ubiquitinate IRF3 leading to its degradation and therefore could be regulating not only the interferon response, but also the production of IFN-α and IFN-β after pathogen recognition [329]. Intriguingly, TRIM21 has been shown to act as an Fc receptor binding IgG via its PRY-SPRY domain [383]. This suggests that it may recognise opsonised viruses or bacteria and may mediate the initiation of an antiviral response via activation of IRF8, but this has yet to be tested.

In addition to effects on the innate immune system several TRIM proteins have been connected to a variety of human diseases often associated with point mutations or the generation of aberrant fusion proteins. Mutations in the PRY-SPRY domain of TRIM20 (pyrin) for example, are the cause of the disease Familial Mediterranean Fever (FMF) [384]. Mutations in the B-box domain of MID1 (TRIM18) are associated with X-linked Opitz G/ BBB syndrome [385] and a mutated form of TRIM32 is found in limb-girdle muscular dystrophy [386]. In some cancers the TRIM27-RET fusion protein acts as an oncogene [387, 388] whereas in cases of acute myeloid leukaemia (AML) TRIM19 is fused to the retinoic acid receptor (RAR) creating a potent oncogene [389]. Other TRIM family members have been associated with autoimmune disorders. In systemic lupus erythematosus (SLE) and Sjögrens syndrome TRIM21 and TRIM68 represent the Ro52 and SS-56 auto-antigens, respectively [390, 391]. In Sjögrens syndrome the antigenic epitopes that are targeted are found in the TRIM21 coiled coil region and the region between the RING and the B-box domain [392]. Direct involvement of many TRIM proteins in controlling virus replication but also the indirect role of TRIM family members on virus propagation by regulating pathogen recognition, and IFN induced signalling pathways highlight the important role this protein family has in innate immunity against viral infections.

1.9. The role of cyclophilins in HIV-1 biology

1.9.1. Cyclophilins are widely expressed PPIases with conserved functions

Cyclophilins (Cyps) are small molecules with an enzymatic activity that enables them to change the isomeric state of peptide bonds at proline residues between *cis* and *trans*. Along with two other protein families, the FK506-binding proteins (FKBP), the parvulins and the recently identified Ser/Thr phosphatase 2A activator (PTPA), cyclophilins are assigned to the group of peptidyl- prolyl- *cis*- *trans*- isomerase enzymes (PPIases). FKBPs and Cyps are sometimes referred to as immunophilins because they can suppress immune responses in a complex with the drugs FK506 or cyclosporine A (CsA), respectively. The PPIase activity of cyclophilins was first demonstrated in 1989 [393]. The enzymatic function of PPIases has been implicated in protein folding of diverse proteins including ribonuclease T1 and collagen [394, 395]. It is believed that PPIases in general accelerate protein folding thereby supporting correct and efficient function of the target protein. In addition, changes in the secondary structure of proteins by isomerisation of peptide bonds may serve as a timed trigger and therefore may be seen as a molecular mechanism to time specific events. The first cyclophilin was discovered in 1984 by affinity purification using a CsA column [396]. PPIase proteins are found in all eukaryotes, prokaryotes and archea and therefore represent an evolutionary conserved repertoire of proteins for diverse functions. Recently, the first virally encoded cyclophilin was described in the Mimivirus but its function remains to be discovered [397].

Cyclophilins are widely expressed and are highly conserved between species [398, 399]. This suggests that they have evolved to perform conserved functions. This is in contrast to the FKBPs PPIases, which differ substantially in their sequences between different species and may have evolved to perform species-specific functions [399]. Humans express at least 17 Cyps including proteins containing cyclophilin like domains, e.g. the nuclear pore protein Nup358, also called RanBP2 or Cyp358. Cyps differ substantially in their sizes ranging from 18kDa for CypA to 358kDa for Nup358. The subcellular localisations of cyclophilins are also very diverse. Human CypA is found in the cytoplasm and the nucleus, CypB is associated with the endoplasmic reticulum, CypC is mainly cytoplasmic, CypD is found in mitochondria, CypE is localised in the cytoplasm, Nup358 is present at nuclear pores and human Cyp19 is localised at the

nuclear spliceosome [399]. Some cyclophilins have N- or C-terminal accessory domains of diverse functions (e.g. the heat shock protein associated cyclophilin Cyp40 possesses three tetratrico peptide repeat (TPR) motifs C-terminal of its Cyp domain; the cytoplasmic cyclophilin CypE has got a RNA binding domain N-terminal of its Cyp domain). It is possible that the function of these domains is to recruit target molecules for enzymatic catalysis by the attached PPIase domain. However, in case of TRIMCyp, a natural occurring fusion protein between CypA and TRIM5, the CypA domain binds to the virus capsid and functions as the recruiting domain, whereas TRIM5 mediates the recruitment of the proteasome (see 1.9.4.).

Cyclophilin A (CypA), formerly referred to as cyclophilin 18 (Cyp18), is an important and probably the most studied member of the cyclophilin family and has been shown to perform diverse roles in numerous biological processes. However, CypA knockout does not impact on the viability of mice or cells, suggesting that functions in the cyclophilin family may be redundant [400, 401]. CypA was demonstrated to modulate signalling of the interleukin-2-tyrosine kinase (Itk). More precisely, CypA mediates a conformational switch in the SH2 domain of Itk resulting in the inhibition of signalling and the increase of PLCgamma1, a downstream substrate of Itk. The role of CypA to switch the conformation and function as a molecular timer has also been proposed for other PPIases and is depicted in FIG 25 [402].

Several cellular interaction partners have been found for CypA. A yeast-two hybrid screen identified the transcription factor YY1 as an interaction partner, suggesting that CypA may also be able to modulate transcription [403]. Retinoblastoma (Rb) protein was also found to interact with CypA and was proposed to interfere with signalling in T-cells [404]. In addition to the direct effect, CypA may also regulate pathways indirectly. Some studies suggest that cellular and virion associated CypA may mediate signalling events in HIV-1 infected cells by binding to different receptors (e.g. CD147 and CXCR-4), however the effects and implications on the virus life cycle remain poorly characterised and understood [405-407]. Furthermore, overexpression of CypA has been observed in different cancers, especially in solid tumours, and has been proposed to confer resistance to hypoxia induced apoptosis, suggesting that perturbing CypA expression levels may contribute to tumorigenesis [408]. Despite many suggested interaction partners, it is not clear how many proteins are isomerised by CypA. However, the consensus sequence FGPXL has been suggested as a binding and isomerisation site and is found in many cellular proteins [409]. The HIV-1 CypA

binding site in CA however has the sequence AGPIA, suggesting some flexibility in the CypA binding motif.

Potentially, due to its multiple cellular interaction partners, CypA has been described as being involved in the life cycles of diverse viruses, including vaccinia virus, vesicular stomatitis virus, mouse cytomegalovirus, hepatitis C virus and influenza virus [410-414]. The effects of CypA on virus replication differ. In some cases CypA acts as a cofactor and stimulates virus replication as seen for example human CypA activity on HCV and HIV-1 replication [400, 412]. In other cases, CypA acts as an inhibitor for virus replication, as seen for example for human CypA activity on influenza A [411]. In contrast to retroviral restriction factors like TRIM5α or APOBEC3G, CypA shows a surprisingly low ratio of non-synonymous to synonymous substitutions per site when human and non-human primate CypA sequences are compared, suggesting that it has not been under positive selection during evolution but rather has been under purifying selection [398].

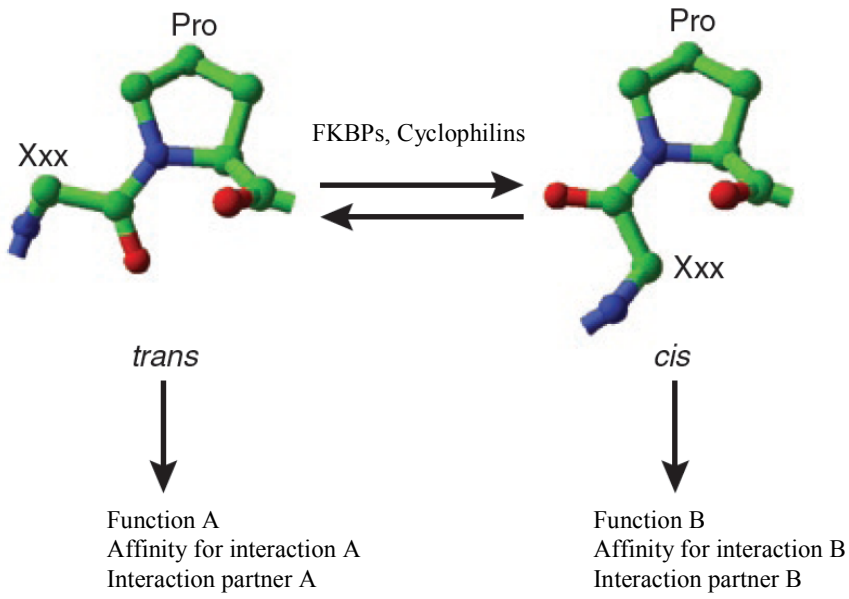


FIG 25 Prolyl-cis-trans-isomerase reaction mediated by cyclophilins and FKBP proteins

Depending on the isomeric state of the proline targeted for isomerisation by Cyps or FKBPs, the protein or domain can exert different functions. In case of TRIM5α restriction, CypA functions as an auxiliary protein. It is possible that different sensitivities for TRIM5α binding can be related to the isomeric state of the targeted proline. Figure adapted from [402].

1.9.2. The PPIase activity of cyclophilins

Cyclophilins and FKBPs perform their enzymatic activity as monomers [415, 416]. The catalytic function of PPIases is to lower the activation barrier and/ or to raise the energy of the bound substrate to the energy of the transition state, in the case of PPIases the 90-*syn* state in which the N-terminal peptide chain is rotated at 90˚ compared to the *cis* or *trans* state. Several mechanisms of how this is accomplished by the enzyme have been proposed, however the exact mechanism is still unknown [402]. One hypothesis is that to interact with the active site of PPIases the substrate is desolvated and liberated from polar interaction partners decreasing the torsion barrier. In addition, intramolecular interactions in the substrate may contribute to lowering the transition barrier. Another theory is that PPIases bind preferentially to the 90-*syn* state and interact less efficiently with the *cis* or *trans* state. In any case, PPIases are able to perform reactions in both directions from *cis* to *trans* and from *trans* to *cis*, however since the free energy of the *cis* state is higher than that of the *trans* state, or in other words the activation barrier for the *trans* to *cis* reaction is higher, reactions from *trans* to *cis* need more energy suggesting that prolyl-peptide bonds in the *trans* state are more prevalent in a protein than the *cis* state.

CypA and other cyclophilins bind to the drug cyclosporine A (CsA) and the structure of human CypA bound to CsA has been solved (FIG 26). CsA is a cyclic polypeptide consisting of 11 amino acids. It is produced as a metabolite by the fungus *Beauveria nivea* [417]. Chemically, CsA is [*R*-[*R**,*R**-(*E*)]]-cyclic(L-alanyl- D-alanyl- *N*-methyl-L-leucyl- *N*-methyl- L-leucyl- *N*-methyl- L-valyl- 3-hydroxy-*N*,4-di-methyl-L-2-amino-6-octenoyl-L-α-amino-butyryl-*N*-methylglycyl-*N*-methyl-L-leucyl-L-valyl-*N*-methyl-L-leucyl). In addition, analogues of CsA are synthesised by the fungus. FKBPs do not bind to CsA but are able to bind to tacrolimus (FK506) and rapamycin (sirolimus). Binding of the drugs to the PPIase active site inhibits its enzymatic function by steric hindrance of substrate binding. CypA consists of eight anti-parallel beta-strands that generate a beta-barrel, which is closed by two alpha helices each at one end of the barrel. CsA as well as the substrate bind to the outer surface of the barrel as shown in FIG 26 and FIG 27. CsA has been widely used as an immunosuppressant and its activity is exerted by a CypA-CsA complex that binds to and inhibits Ser/ Thr phosphatase 2B (PP2B), also known as calcineurin [418]. FK506 bound to FKBPs functions in a similar way, whereas binding of rapamycin to FKBPs leads to the inhibition of the target of rapamycin (TOR), a protein involved in diverse processes including cell division and

growth [419]. Calcineurin targets the nuclear factor of activated T-cells, NF-AT, for dephosphorylation, thereby inducing its nuclear translocation and allowing the initiation of transcription of genes mediating T-cell activation or proliferation, e.g. IL-2 [420]. Inhibition of calcineurin prevents normal T-cell activity and this has been used for example to suppress rejection of organs after transplantation.

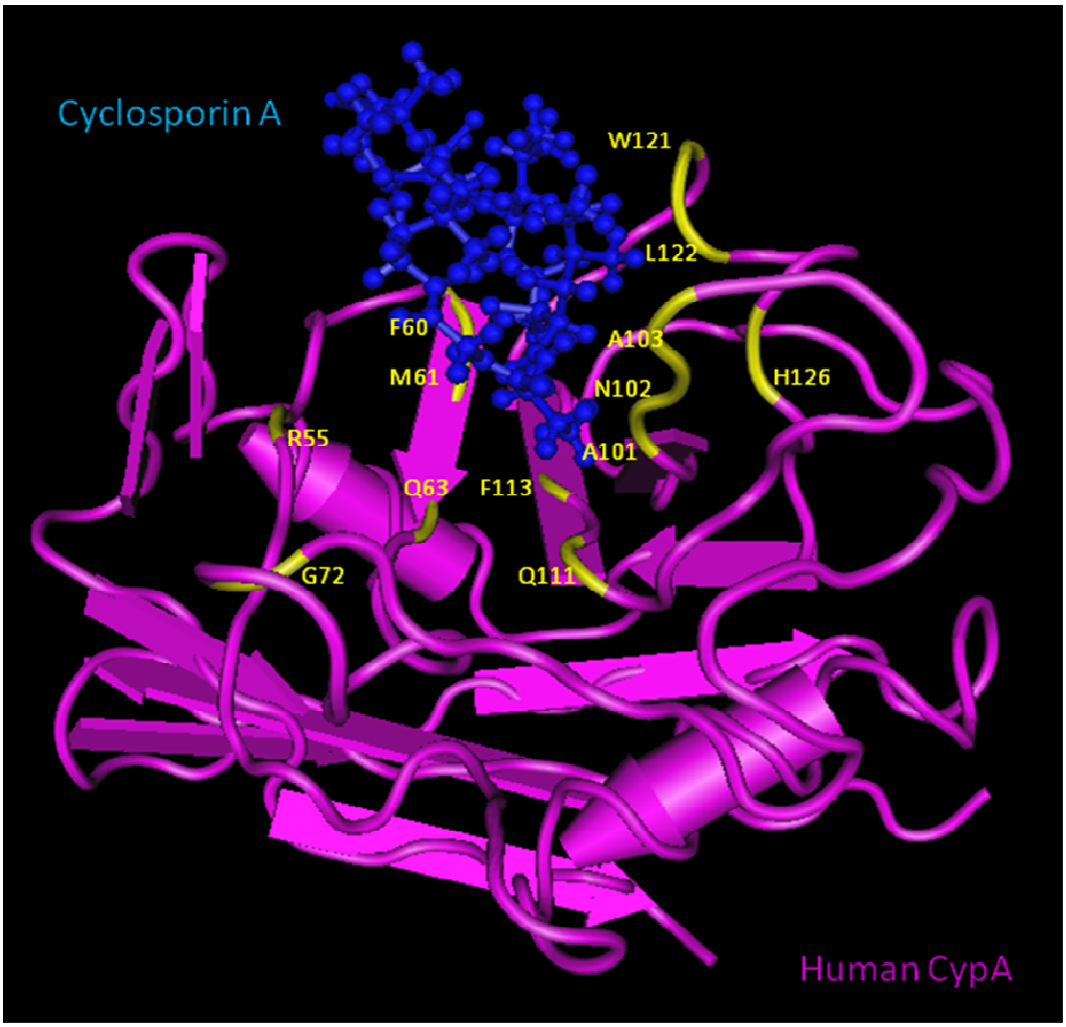


FIG 26 NMR structure of human CypA complexed with cyclosporine A

The NMR structure of human cyclophilin A (CypA) (purple) complexed with the drug cyclosporine A (blue). The structure was obtained from PDB file 3CYS and analysed using the program Cn3D4.1. CypA amino acid residues important for CsA binding are indicated in yellow [421].

1.9.3. CypA's species-specific activity on HIV-1

Cyclophilin A and B have been found to bind HIV-1 Gag, originally by a yeast-two hybrid screen [422]. This interaction was then mapped to the CA portion of Gag, specifically to the G89-P90 peptide bond [423].

Different X-ray structures of CypA complexed with HIV-1 N-terminal CA fragments demonstrated, that CypA binds to and isomerises the peptide bond between glycine G89 and proline residue P90 and rotates the N-terminal peptide chain, whereas the C-terminal peptide chain stays anchored (FIG 27) [424]. The G89-P90 peptide bond is found in the *cis* state in 14% of analysed NMR structures and the *trans* state in 86%, which is in agreement with the higher transition barrier for the *trans* to *cis* reaction (see 1.9.2.) [425]. Interestingly, changing CA residue A88 to methionine leads to a change in the state of P90 from *trans* to *cis* [424]. CA residue M88 is found in most HIV-1 O-group viruses, whereas M-group HIV-1 isolates have different amino acids at this position (Greg Towers, unpublished). This difference in the isomeric state of the peptide bond between G89 and P90 may explain the opposing sensitivities of HIV-1 M-group and O-group viruses to restriction by rhesus macaque TRIMCyp (see 1.9.4.). P90 is located in a proline rich loop in the N-terminal part of HIV-1 CA facing the exterior of the capsid shell allowing the interaction with CypA [426].

Due to its Gag binding ability, CypA is packaged into newly produced HIV-1 virions at an approximated ratio of one CypA molecule per ten CA molecules [426, 427]. Some studies suggested that membrane exposed CypA in the virions is involved in virus entry by interaction with cellular heparins however this model was not supported by more recent observations [428, 429]. Analysis of virion associated CypA showed that in addition to wild type CypA, N-terminal modified forms may be incorporated into virions [430, 431]. The functions of virion associated CypA and its different isoforms however remain unknown. CypA interaction with Gag and packaging can be abrogated by mutating the proline residue (P90A) or neighbouring residues (G89V), or by addition of CsA [426, 427].

Cyclophilin A functions as a co-factor for HIV-1 replication in human cells, whereas in most non-human cells CypA has a negative effect on HIV-1 infection. Decrease of CypA activity in human cells by siRNA mediated knock down or CsA treatment also decreases replication of HIV-1 and this appears to be in a TRIM5α independent manner [432, 433].

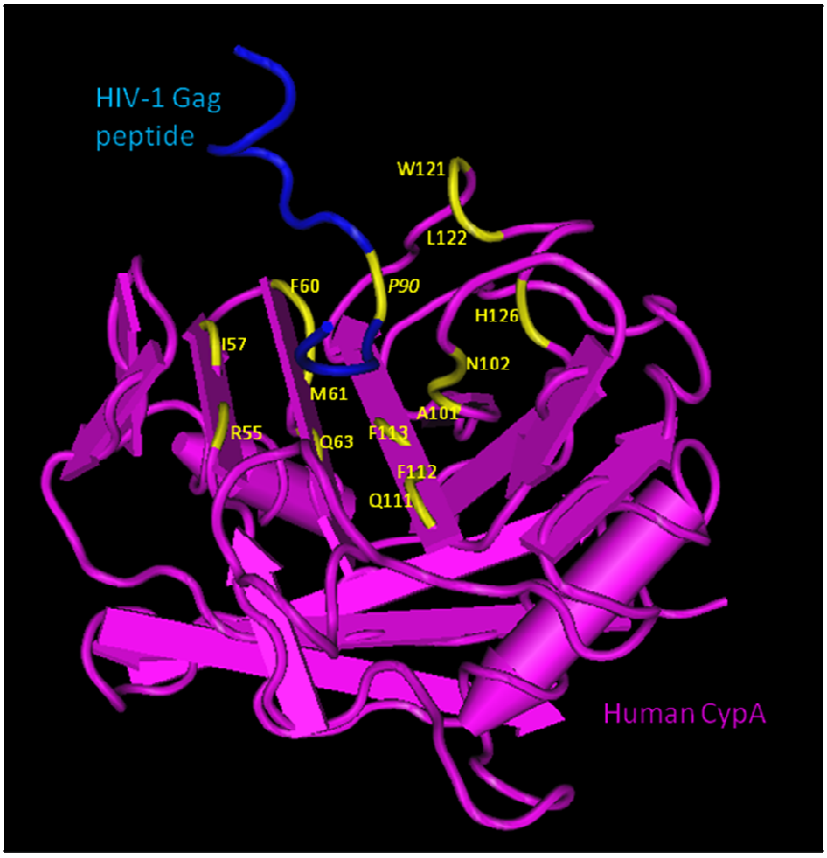


FIG 27 Structures of CypA complexed with an HIV-1 Gag peptide

Structure was obtained from PDB file 1FGL and shows an HIV-1 Gag peptide fraction bound to the active site of human CypA. HIV-1 CA peptide chain is shown in blue and P90 is shown in yellow italic. Human CypA is shown in purple. Important CypA sites involved in substrate binding are labelled in yellow [421]. The picture was obtained using the structure analysing software Cn3D4.1.

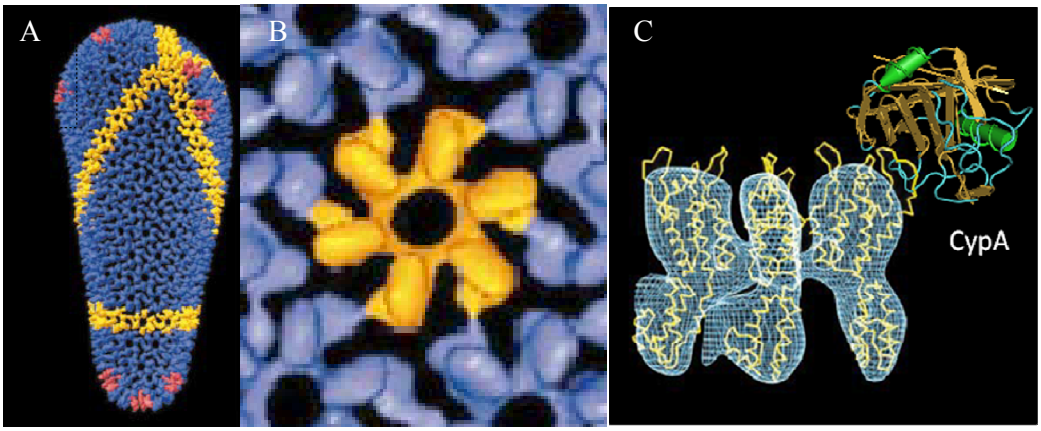


FIG 28 CypA interaction with the HIV-1 core

A) The HIV-1 core consists of hexameric and pentameric CA structures. B) CA hexamer enlarged. C) CA hexamer from the side. Extruding loops are the interaction sites for CypA, which was superimposed to the hexameric CA structure. Figures A-C adapted from [434].

Indeed, it has been demonstrated that the target cell CypA rather than the virion associated CypA impacts on HIV-1 infectivity [435-438]. In this model, target cell CypA interacts with incoming viral cores and confers enhanced sensitivity to cellular restriction factors. Restriction factors, like TRIM5α, may preferably bind to a certain conformation of the G89-P90 bond either *cis* or *trans*. CypA catalyses the isomerisation of this bond thereby altering capsid conformation. The presence of CypA may therefore support the availability of peptide bonds for TRIM5α binding. This model is very attractive since it may explain the species-specific phenotypes seen for inhibition of CypA in divergent cells by the presence of different co-factors or restriction factors with differential preferences for the *cis* or *trans* state of the G89-P90 bond [439].

In addition to HIV-1, SIVcpz also binds CypA [440]. Different results have been obtained for SIVmac incorporation of CypA [440, 441]. Interestingly, the transfer of the cyclophilin binding site from HIV-1 into the CA protein of SIVmac can confer the sensitivity to inhibition by cyclosporine in human cells [442]. More and more evidence suggests that many lentiviruses interact with CypAs from different species. Indeed, a variety of lentiviruses including HIV-2 and SIVmac possess the conserved proline CA residue found in HIV-1 (P90) [439]. CA proteins of the two divergent lentiviruses feline immunodeficiency virus (FIV) and SIVagmTan, have also been reported to associate with human CypA [342, 343, 443]. Indeed, FIV as well as SIVagmTan are targets for restriction by the naturally found owl monkey TRIMCyp protein (see 1.9.4.), suggesting that monomeric owl monkey CypA can also bind to their capsids [319]. Treatment of feline CRFK cells with CsA during FIV infection decreases infectivity in a dose dependent manner, suggesting that CypA activity of a certain species to viruses that circulate in this species is conserved (e.g. human CypA is a co-factor for HIV-1 in human cells) [444]. In addition, HIV-2 weakly binds human CypA and is targeted by the second naturally occurring TRIMCyp protein from macaques (see 1.9.4.) [186]. However, CsA treatment seems not to decrease HIV-2 infectivity in human cell lines, suggesting that CypA at least, is not important for HIV-2 replication.

When HIV-1 is cultured in CD4+ HeLa cells in the presence of CsA, mutant viruses arise and the mutations can be mapped to HIV-1 CA (A92E, G94D, R143K) [445]. Interestingly, mutant viruses are poorly infectious (only ~ 10 % of wild type HIV-1) in the absence of cyclosporine but infectivity is rescued when CsA is present during infection [436, 446]. In addition, in the absence of cyclosporine mutant viruses quickly revert to wild type virus or generate suppressor mutations in CA (V86A, A88T) [445].

Recent studies showed that the HIV-1 CA protein might play a role in nuclear import of HIV-1 pre-integration complexes (see 1.4.3.) [72]. When HeLa cells are arrested with aphidicolin, a drug that binds to DNA polymerase II and blocks cell division in the early S-phase, infectivity of HIV-1 wild type virus is not changed whereas the infectivity of the CsA CA escape mutants HIV-1 A92E or G94D is further decreased but can be fully rescued by addition of CsA [81, 447].

The situation in non-human cells seems to reflect the situation seen for the CsA resistant mutant HIV-1 viruses in human cells, in that depletion of CypA increases infectivity of the virus. In fact, studies have suggested that CypA acts as an auxiliary factor to HIV-1 restriction by Old World Monkey TRIM5α, although restriction in the absence of CypA is only moderately diminished [433, 448, 449]. In contrast to humans, decreasing cellular levels of CypA using siRNA or CsA increases the infectivity of HIV-1 in Old World monkey cells. However, whether restriction depends on the enzymatic activity of CypA causing a changed sensitivity of CA to recognition by TRIM5α, as proposed by the above model, or whether CypA functions as a molecular inhibitor to a putative co-factor, is not clear [450].

1.9.4. TRIMCyps

Remarkably, in cells from the New World owl monkey (*Aotus*), CypA has been fused to TRIM5α replacing the viral binding B30.2 domain and resulting in a fusion protein referred to as TRIMCyp [187, 451]. Owl monkey TRIMCyp restricts HIV-1, SIVagm and FIV via recruitment of the CypA domain to the viral capsid [187, 319]. Inhibition of the interaction between CypA and capsid using CsA, rescues HIV-1, SIVagm and FIV infectivity in owl monkey cells. Surprisingly, a second TRIMCyp fusion protein has been identified in rhesus macaques (*Macaca mulatta*), pigtailed macaques (*Macaca nemestrina*) and cynomolgus macaques (*Macaca fascicularis*) [35, 186, 452]. Interestingly, TRIMCyp from macaques does not restrict HIV-1 but restricts HIV-2 and FIV, indicating differences in capsid recognition. The discovery of two fusion proteins of TRIM5 with CypA in *Aotus* and *Macaca* suggests an important role of both, TRIM5 and CypA, in providing a selective advantage when they fuse. However, it is not known what the selective advantage is. Both TRIMCyp fusion proteins have evolved independently by LINE-1 mediated retrotransposition of the CypA cDNA into two different positions in the TRIM5 locus (FIG 29). In the case of TRIMCyp from owl

monkeys, the CypA coding region is found inserted into intron 7 of TRIM5 and an aberrant splicing of exon 7 to the CypA pseudogene generates the TRIMCyp fusion protein. In the case of macaque TRIMCyp, the CypA cDNA inserted downstream of exon 8. A disrupted splice acceptor site in exon 7 causes an aberrant splicing of exon 6 to the CypA coding region downstream of exon 8, generating the macaque TRIMCyp fusion protein.

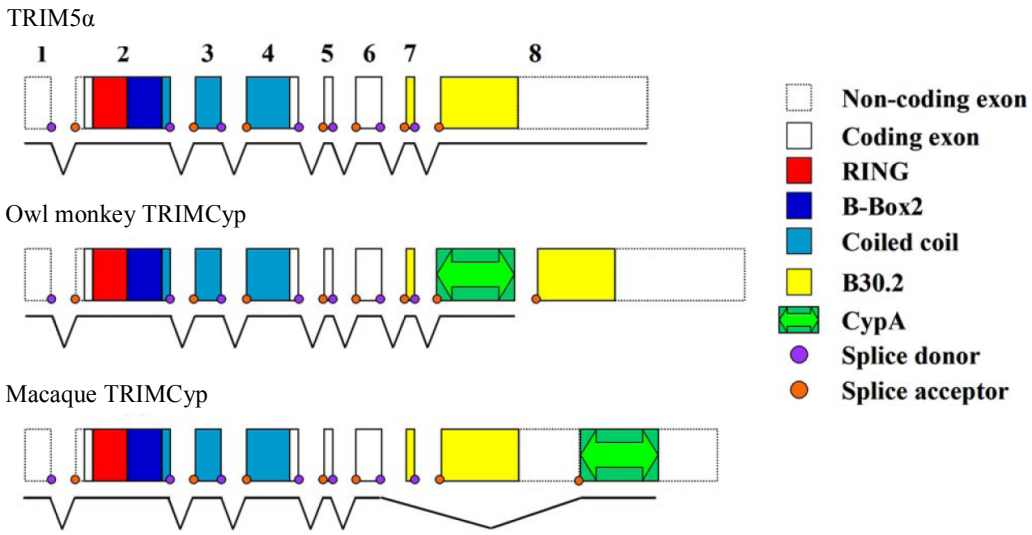


FIG 29 Evolution of TRIMCyps by retrotransposition of CypA

Many mammals possess a TRIM5 gene that encodes for a TRIM5α protein, which possesses a C-terminal B30.2 domain (yellow). In owl monkeys and some macaque species, a CypA mRNA was retrotransposed and inserted into intron 7 (owl monkeys) or downstream of exon 8 (macaques), leading to the expression of TRIMCyp fusion proteins. In case of owl monkey TRIMCyp the CypA splice acceptor replaces the exon 8 splice acceptor leading to a fusion of exon 7 to the CypA coding sequence, whereas in macaque TRIMCyp exon 7 does not possess a correct splice acceptor leading to exon 7 and 8 skipping. Figure adapted from [453].

1.10. Experimental system to study early events of retroviral infections

The experimental approach relies on the use of retroviral vectors derived for gene therapy. Pantropic VSV-G pseudotyped virus like particles with a virus core derived from the GagPol expression plasmid and the viral vector RNA encoding GFP are produced and used to infect target cells. Viral RNA is reverse transcribed and proviral DNA is integrated into the target cell genome. Formation of the provirus results in the expression of GFP which is measured by fluorescence activated cell sorting (FACS). Alternatively, levels of different viral reverse transcription products generated in the infected target cell (e.g. *GFP*, 2-LTR circles) are measured by Taqman quantitative PCR.

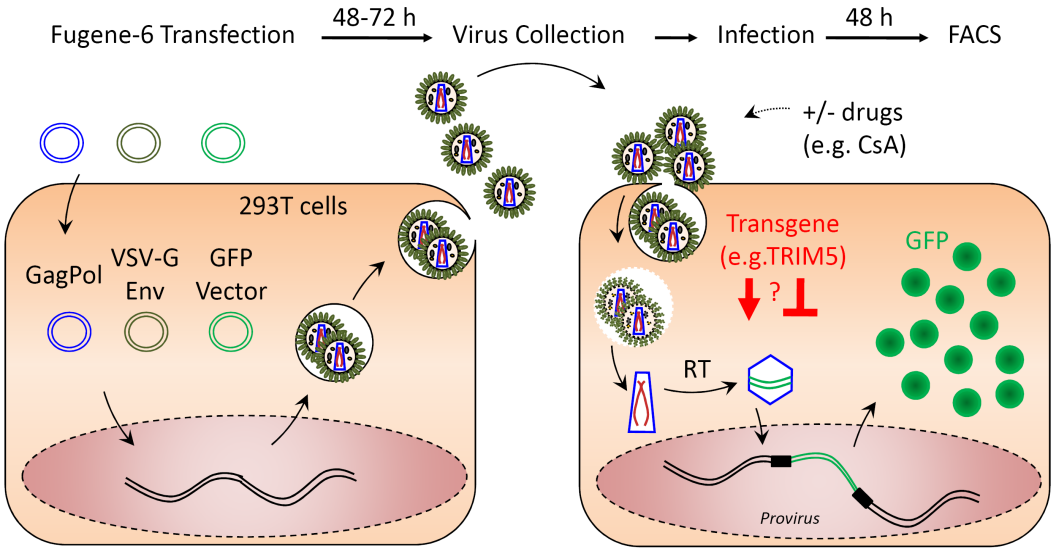
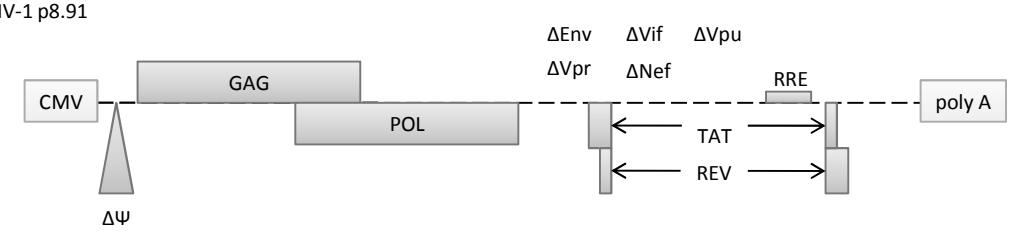


FIG 30 The virus vector system to study the HIV-1 life cycle

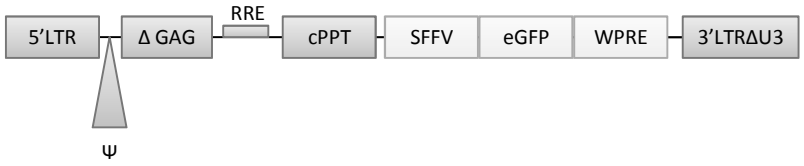
293T cells are transfected with three plasmids expressing VSV-G envelope protein, GagPol protein and the vector RNA encoding for GFP. Pantropic virus-like particles are used to infect different target cells and infection is measured by fluorescence activated cell sorting (FACS). Alternatively, reverse transcription (RT) products are measured by Taqman quantitative PCR. The effect of a transgene (e.g. TRIM5α) can be tested by measuring percent of cells that become GFP positive. In addition, the effect of drugs (e.g. CsA) on infection can be monitored.

1.11. Vector maps of the different retroviruses used in the study

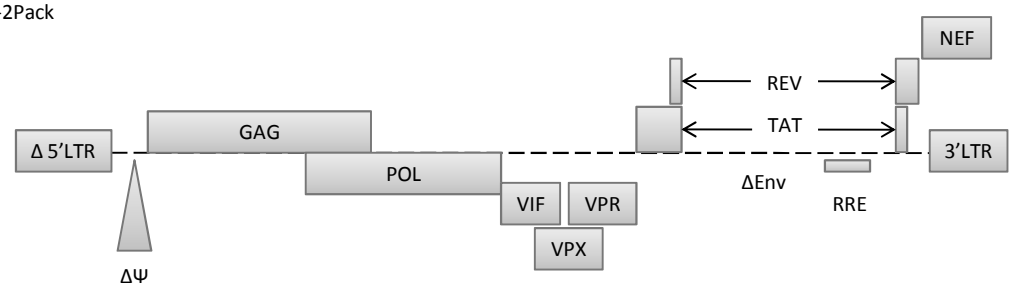
HIV-1 p8.91



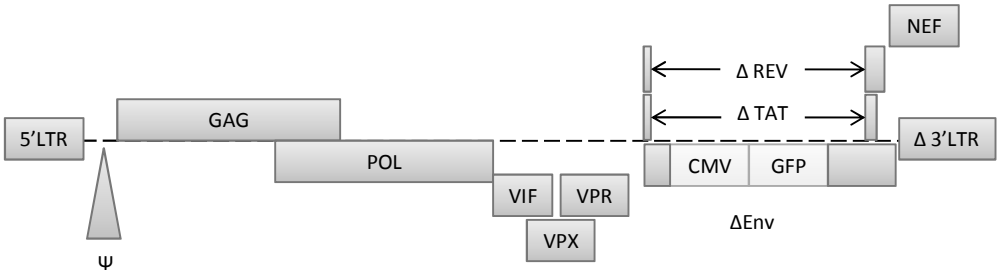
HIV-1 pCSGW



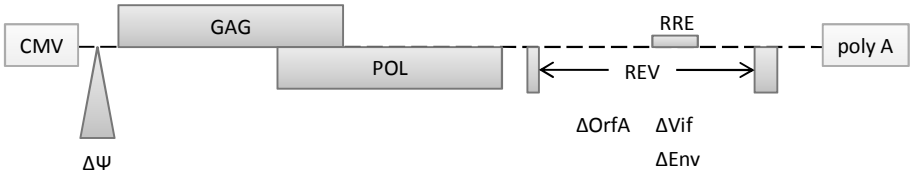
HIV-2Pack



HIV-2GFP

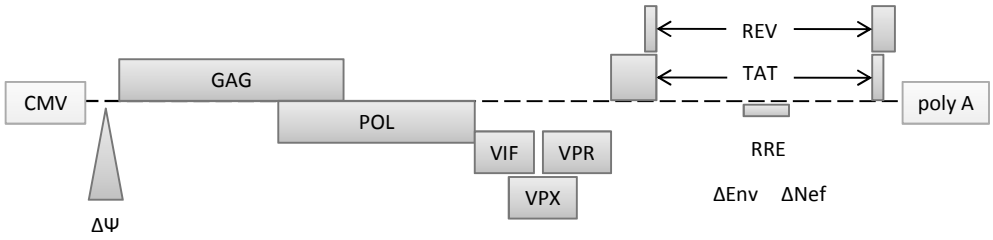


FIV pFP93

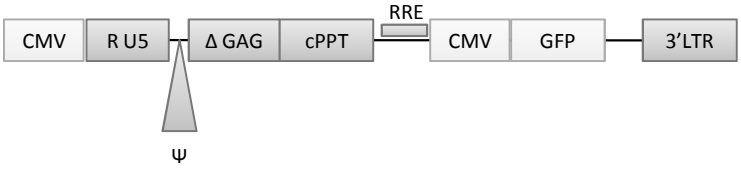


FIV pGinSin

SIV pSIV3+



SIV GFP (pSIV RMES4)



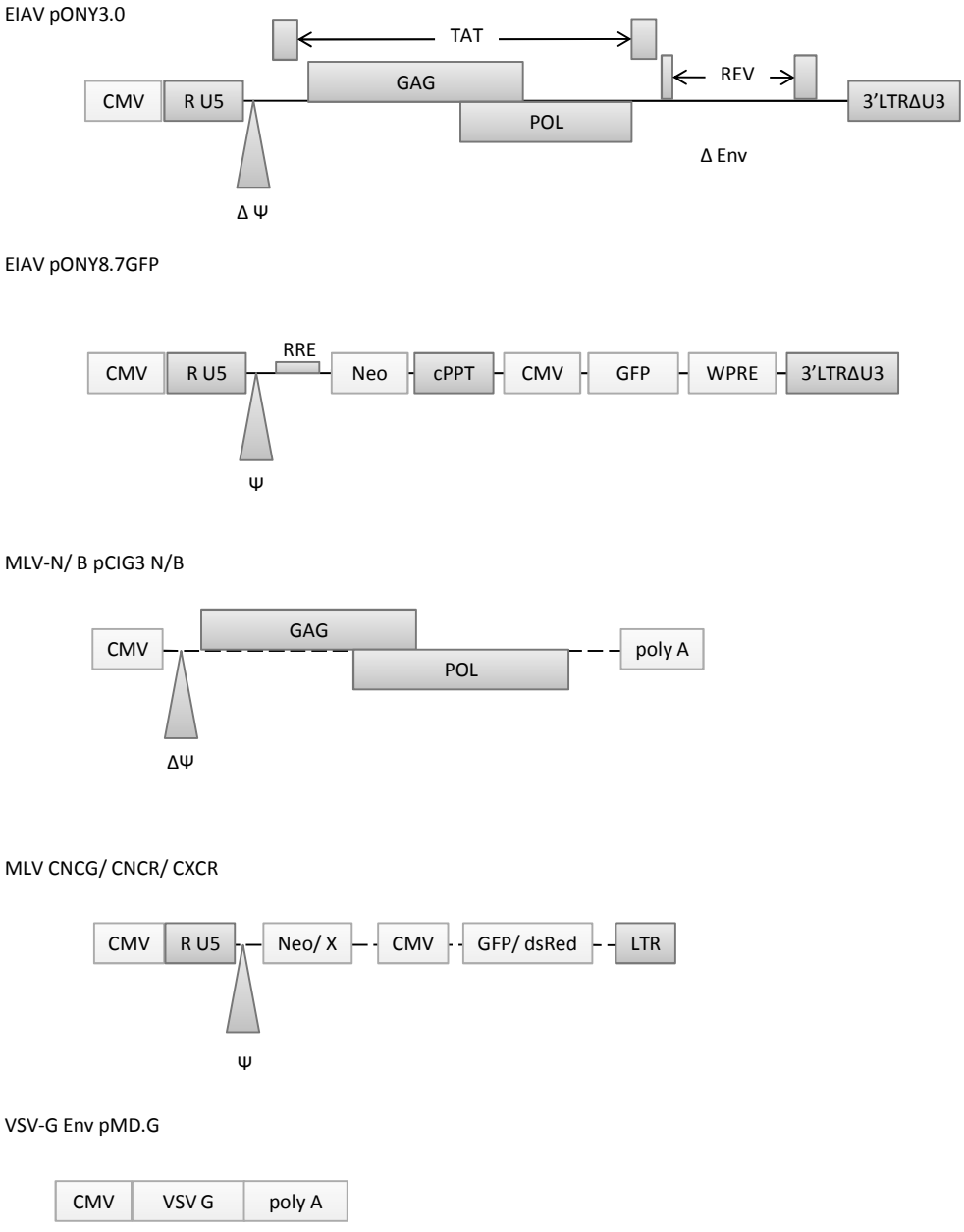


FIG 31 Retroviral packaging and vector maps

Vector maps were derived from HIV-1 [454, 455], HIV-2 [456], FIV [457], SIVmac [458], MLV [286, 459], EIAV [460, 461]. Dark grey boxes are viral elements and light grey boxes are non-viral elements. CMV, cytomegalovirus promoter; Ψ, RNA packaging signal; RRE, Rev responsive element; cPPT, central poly purine tract; Neo, neomycin resistance gene; WPRE, woodchuck hepatitis virus post-transcriptional regulatory element; SFFV, spleen focus forming virus promoter; IRES, internal ribosome entry site; VSV-G, vesicular stomatitis virus G protein; LTR, long terminal repeat

2. Materials and Methods

2.1. Materials

2.1.1. Cells

Bacteria

HB101: F^- mcrB mrr hsdS20(r_B^- m_B^-) recA13 leuB6 ara-14 proA2 lacY1 galK2 xyl-5 mtl-1 rpsL20(Sm^R) glnV44 λ^- described in [462]

Mammalian cell lines

CRFK (Crandell Reese feline kidney cells; gift from Y. Ikeda) described in [463]

- CRFK cells do not express an antiviral TRIM5α protein and have been demonstrated to be permissive for the infection with retroviral vectors used in this study. They were therefore used for exogenous expression of the restriction factors analysed.

SIRC (Statens Serum institute rabbit corneal cells; gift from Y. Takeuchi) described in [464]

EREP (Epithelial rabbit cell line; Centro Substrati Cellulari Brecia, CSCB)

TE671 (Human rhabdomyosarcoma cells, gift from Y. Takeuchi) [465]

293T (Human embryonic kidney cells, original name: 293tsA1609neo, gift from Stephen P. Goff) [466, 467]

OMK (Owl monkey kidney cells, derived from ECACC) [468]

HeLa (Human epithelial cells from cervical carcinoma, gift from Ariberto Fassati) [469]

HareKF (Hare kidney fibroblast cell line; gift from Jean Francois Vautherot)

2.1.2. Media

Bacteria

LB-Medium: 10 g tryptone, 5 g yeast-extract, 5 g NaCl per 1 l medium; ampicillin was added at a concentration of 100 µg/ml. Solid medium plates were generated by adding 1.5 % agar-agar to the liquid medium.

Cell cultures

Cell monolayers of all cell lines were routinely grown in Dulbecco's modified eagle's medium (DMEM) (GIBCO, Invitrogen, Paisley, UK) supplemented with 100 U/ml of penicillin and 100 µg/ml of streptomycin (GIBCO, Invitrogen, Paisley, UK), and usually 10 % fetal bovine calf serum (FBS) (BioSera, Ringmer, UK). Suspension T-cell lines were normally grown in RPMI (GIBCO, Invitrogen, Paisley, UK) containing 10 % FBS. 293T cells were usually grown in DMEM containing 15 % FBS.

2.1.3. Buffers, solutions and drugs

10×TBS: 5 litres contain 120.5 g Tris, 400 g NaCl, pH 7.6

10×SDS PAGE running buffer: 5 litres contain 50 g SDS, 150 g Tris, 720 g glycine

Protein sample buffer: 0.2 M Tris HCl pH 6.8, 5.2 % SDS, 20 % glycerol, 0.1 % bromphenol blue, 40 mg/ml DTT on the day

Semiphor Transfer buffer: 25 mM Tris, 192 mM glycine, 20 % methanol

NiNTA Buffer A: 6 M guanidine-HCl; 0.1 M Na_2HPO_4/NaH_2PO_4; 10 mM imidazole; pH 8.0

NiNTA Buffer TI: 25 mM Tris HCl; 20 mM imidazole; pH 6.8

NiNTA Buffer A/TI: 1 volume NiNTA Buffer A + 3 volumes NiNTA Buffer TI

Buffer TfB1: 30 mM KAc, 100 mM RbCl, 10 mM CaCl, 50 mM MnCl, 15 % glycerol in H_2O, sterilised with 0.45 µm filter

Buffer TfB2: 10 mM PIPES, 75 mM $CaCl_2$, 10 mM RbCl, 15% glycerol in H_2O, sterilised with 0.45 µm filter

Ethidiumbromide (10 mg/ ml): Sigma Aldrich, Dorset, UK; used at 0.5 µg/ ml

DNA loading buffer: 5× GoTaq Buffer with loading dye, Promega Southampton, UK

Cell freezing mix: Fetal bovine serum containing 10 % DMSO

OptiMEM for transfections: GIBCO, Invitrogen, Paisley, UK

Aphidicolin (2 mg/ ml in DMSO): Calbiochem, Merck Chem. Ltd., Nottingham, UK

MG132 (1mg/ ml in EtOH): Sigma Aldrich, Dorset, UK; used at 5-8 µg/ ml

Cyclosporine (Sandimmune) (50 mg/ ml): Sandoz Pharmaceuticals, Camberley, UK

Polybrene (8 mg/ ml in H_2O): Sigma Aldrich, Dorset, UK; used at 8 µg/ ml

Ampicillin (100 mg/ ml): Calbiochem, Merck Chemicals Ltd., Nottingham, UK; used at 0.1 mg/ ml

Kanamycin (30 mg/ ml): Sigma Aldrich, Dorset, UK; used at 30 µg/ ml

G-418 (100 mg/ ml in H_2O): Calbiochem, Merck Chemicals Ltd., Nottingham, UK; used at 1 mg/ ml

Puromycin (2.5 mg/ ml in H_2O): Sigma Aldrich, Dorset, UK; used at 1-7 µg/ ml

2.1.4. Primers

TS1	GGAGGAGGTGACCTGTCC
TS2	TGTGGCCACAGCTTCTGCCAAG
TS6	TCAGGGAAACATTACTGGG
TS8	CTTGGCAGAAGCTGTGGCC
TS10	GAGCAGGAGTTTCTCTCCATG
TS12	CCCAGTAATGTTTCCCTGA
TS16	CATAGTCTAGGAAAACTCCAACACG
TS20	ATCGGAATTCCACCATGGCTTCAGCAATCTTAGCGAATATGAAG
TS22	TAGCTTCGAATCAACAGCTCAACTCGCAGATTGTCATGG
TS24	CGATGCGGCCGCTCAAGAGCTTGGTGAGCACAGAG
TS27	CTGCAGACGCGTGACACCCTG
TS28	GTCACGCGTCTGCAGCTCTCTTG
TS29	GGATTAAAACGCGTAGGGAACTTG
TS30	CCCTACGCGTTTTAATCCCATC
TS31	GTTTAACGCGTACAGCAGCTAC
TS32	GCTGTACGCGTTAAACTTAATTC
TS33	TAGCACGCGTATGGTCAATCCTACTGTGTTC
TS34	CGATTTCGAATTAAAGTTGTCCACAGTCAG
TS35	CAGTGAATTCATGGCTTCAGCAATCTTAGCGAATATG
TS36	CGATGCGGCCGCTCAACAGCTCAACTCGCAGATTGTC
TS41	GTGGGGGCAGCGTCTGTCCTGTGTGCCGGATCAGTTACCAGCCTGAGAACATAC
TS42	GTATGTTCTCAGGCTGGTAACTGATCCGGCACACAGGACAGACGCTGCCCCCAC
TS43	CAGTGAATTCATGGCTTCAGCAGCACGCTTGACAATGATG
TS44	GAGGAAAAAGCAGCTGTCCTGTGTGTCGGATCAGTTACCAGCCTGAGAACATAC
TS45	GTATGTTCTCAGGCTGGTAACTGATCCGACACACAGGACAGCTGCTTTTTCCTC
TS46	CTCAGGAGCACCGTGGTCACCACACATTCCTCACAGAGGAGGTTGCCCGGGAG
TS47	CTCCCGGGCAACCTCCTCTGTGAGGAATGTGTGGTGACCACGGTGCTCCTGAG
TS48	CATCAGAGAAGAGAAAACTTCCTGGAAGACTCAAATACAGTATGACAAAACCAACG
TS49	CGTTGGTTTTGTCATACTGTATTTGAGTCTTCCAGGAAGTTTTCTCTTCTCTGATG
TS50	CAAAGGAGAGAGTAGCTGCCCTGTGTGCGGTATCAGTTACTCATTTGAACATCTAC
TS51	GTAGATGTTCAAATGAGTAACTGATACCGCACACAGGGCAGCTACTCTCTCCTTTG
TS52	GTCTCAGGAGCACCGTGGTCACCACACGGTCCTCACGGAGGAAGTATTCAAGGAATG
TS53	CATTCCTTGAATACTTCCTCCGTGAGGACCGTGTGGTGACCACGGTGCTCCTGAGAC
TS54	CATCAGAGAAGAGAAAGCTTCCTGGAAGTATCAGGTACAAACTGAGAGACAAAGG
TS55	CCTTTGTCTCTCAGTTTGTACCTGATACTTCCAGGAAGCTTTCTCTTCTCTGATG
TS56	GCTACAATTGATGGCTTCAAAAATCTTGCTTAACGTAC
TS57	TACGGCGGCCGCTCAAGAGCTTGGTGGGCATAGAGTCATG
TS58	ATGCCAATTGATGGCTTCTGGAATCCTGGTTAATGTAAAGG
TS65	GATCCGTCCAAAGCCCATGACAATTTCAAGAGAATTGTCATGGGCTTTGGACTTTTTTACGCGTG
TS66	AATTCACGCGTAAAAAAGTCCAAAGCCCATGACAATTCTCTTGAAATTGTCATGGGCTTTGGACG
TS69	GATCCACCACACCTACTCCAGATATTCAAGAGATATCTGGAGTAGGTGTGGTTTTTTTACGCGTG
TS70	AATTCACGCGTAAAAAAACCACACCTACTCCAGATATCTCTTGAATATCTGGAGTAGGTGTGGTG
TS77	CAGACGAATTCCACCATGCATTGTGCACGCCATGGAGAGAAACTCCTAC
TS82	CATACAAATAACTACATCATGTCCC
TS83	GTTAAAATGGTACAGATGGGCTTAG
TS84	CATGGAATTCCGGATCAGTTACCAGCCTGAG
TS85	GCTAGCGGCCGCTTAAAGTTGTCCACAGTCAGCAATGGTG

TS86	GACTGAATTCGAGGAAAGTGTGGTGACCACGGTG
TS90	ATCGGAATTCGATCACTGTGCACGCCATGGAGAGAAAC
TS91	GATCGAATTCACTTTCCTCATGGAGGAGGTTGC
TS93	GATCGAATTCACGTTCCTCACAGAGGAGGTTGC
TS127	GATCGAATTCATGGCTTCCAGAATCCTGGTCAATATAAAGG
TS128	CGATGCGGCCGCGGCTGATGCTACAAGGTCCCAGGCG
TS129n	GCATGCGGCCGCAGAATTATCAAAGGAGACCAATCCTGTGGTG
TS130	ATGCGTCGACTTATATCTGTCCACATTCTGTGATAG
TS131n	GCATGCGGCCGCCATGAAGGTGCTCCTTGCCGCCGCCCTCATC
TS132	GCATGTCGACCTACTCCTTGGCGATGGCAAAGGGCTTCTCC
TS133	GCATGCGGCCGCATGGGCCCGGGTCCTCGGCTGCTGCTACCTC
TS133n	GCATGCGGCCGCCATGGGCCCGGGTCCTCGGCTGCTGCTACCTC
TS134	GCATGTCGACTCACCAATCAGCGATCTCAACCACAAAAGG
TS135	GCATGCGGCCGCATGGCCACCACCAAGCGCGTCTTGTACGTG
TS135n	GCATGCGGCCGCGATGGCCACCACCAAGCGCGTCTTGTACGTG
TS136	CGATGTCGACTCACACGTACTCCCCACAGTCGGCGATGATC
TS137	GCATGCGGCCGCATGCTGGCGCTGCGCTGCGGCTCCCGCTGG
TS137n	GCATGCGGCCGCGATGCTGGCGCTGCGCTGCGGCTCCCGCTGG
TS138	ATGCGTCGACTTAGCTCAACTGGCCACAGTCTGTGATGAC
TS141	GCATGCGGCCGCCATGGTCAACCCCACCGTGTTCTTCG
TS142	GCATGTCGACTTATTCGAGTTGTCCACAGTCAGC
TS143	CATGGAATTCCACCATGCGGATCAGTTACCAGCCTGAGAACATACAGC
TS154	AGCTGCGGCCGCTGTCCTCAGCATCTTCTTCAGCCCTGGCAC
TS155	AGCTGCGGCCGCAGTGATGTGGACTGCACATGTCCTCAGCATC
TS156	AGCTGCGGCCGCCACTTGTCTCCGATCTTCTGAAAGTATC
TS158	CATGGAATTCCACCATGCGGATCAGTTACCAGCCTGAG
TS159	CATGGAATTCCACCATGACTTTCCTCATGGAGGAGGTTGC
TS183	AGCCGCGGCCGCCATGGTCAATCCTACTGTGTTC
TS184	GACTGTCGACTTAAAGTTGTCCACAGTCAGC
TS185n	ATGCCAATTGCGGATCAGTTACCAGCCTGAGAACATACAGCC
TS208	GCATCTCGAGTTAGAGTTGTCCACAGTTGGCAATG
GT270	ATCGTTCGAATCAAGAGCTTGGTGAGCACAGAG
GT273	ATCGGCGGCCGCTCAAGAGCTTGGTGAGCACAGAG
GT389	GATCCAGTAGAACCCCTGAGCATATTCAAGAGATATGCTCAGGGGGTTCTACTTTTTTTACGCGTG
GT390	AATTCACGCGTAAAAAAAGTAGAACCCCTGAGCATATCTCTTGAATATGCTCAGGGGTTCTACTG
GT391	GATCCGCGGTTACCAACTTGAGAACTTCAAGAGAGTTCTCAAGTTGGTAACCGTTTTTTACGCGTG
GT392	AATTCACGCGTAAAAAACGGTTACCAACTTGAGAACTCTCTTGAAGTTCTCAAGTTGGTAACCGCG
GT437	GATCGAATTCATGGCTTCTGGAATCCTGCTTAATG
GT471	GATCGAATTCCGGATCAGTTACCAGCCTGAGAAC
GT472	ATCGGAATTCGATCACTGTGCACACCATG
GT473	GATCGAATTCACATTCCTTGTGGAGGAGGTTGC
GT474	GATCGCGGCCGCTTAAAGTTGTCCACAGTCAGC
GT487	GATCGCGGCCGCTTATTCGAGTTGTCCACAGTCAGC
SWJ35	GATCGAATTCATGGCTTCTGAAATCCTGCTTAATG
TY2	ATAGCGGCCGCTGGTGAGAGATGGGTGCGAGAGCGTCAGTATTA
TY3	CTATGAGTATCTGATCATACTACTGTCTTACTTTGATAA

Table 2 Oligonucleotide sequences used in this study

2.1.5. TaqMan qPCR primer/ probes/ standards

Target	Primer 1 5'-3'	Primer 2 5'-3'	Probe 5'-3' (F: FAM, Ta:TAMRA)	Standard
GFP	GT139 (CAACAGCCACAACGTCTATATCAT)	GT140 (ATGTTGTGGCGGATCTTGAAG)	GFP-P (**F**)-CCGACAAGCAGAAGAACGGCATCAA-(**Ta**)	pCNCG
LRT-P	MH531 (TGTGTGCCCGTCTGTTGTGT)	MH532 (GAGTCCTGCGTCGAGAGAGC)	LRT-P (**F**)-CAGTGGCGCCCGAACAGGGA-(**Ta**)	pCSGW
***2-LTR* circles**	MH535 (AACTAGGGAACCCACTGCTTAAG)	MH536 (TCCACAGATCAAGGATATCTTGTC)	MH603 (**F**)-ACACTACTTGAAGCACTCAAGGCAAGCTTT-(**Ta**)	pGEM-2LTR1
***2-LTR* circles**	2-LTR LA Fwd (AACTAGAGATCCCTCAGACCCTTTT)	2-LTR LA Rev (CTTGTCTTCGTTGGGAGTGAATT)	2-LTR LA P (**F**)-CTAGAGATTTTCCACACTGAC-(**Ta**)	p2LTR (LA)

Table 3 TaqMan qPCR primer/ probes/ standards

2.1.6. Antibodies

Name	Antigen	Company	Species	Application
3F10	HA	Roche Applied Science, Burgess Hill, UK	mouse, monoclonal, HRP linked	1:5000 in WB
SA296	CypA	BioMol, Exeter, UK	rabbit, polyclonal	1:3000 in WB
183	HIV-1 CA/p24	NIH, Bethesda, USA	mouse, monoclonal	1:1000 in WB
AC-15	betaActin	Abcam plc, Cambridge, UK	mouse, monoclonal	1:40000 in WB
ECL Mouse IgG	mouse IgG	GE Healthcare, Little Chalfont, UK	sheep, polyclonal HRP linked	1: 5000 in WB
ECL Rabbit IgG	rabbit IgG	GE Healthcare, Little Chalfont, UK	donkey, polyclonal HRP linked	1:5000 in WB
HA.11 16B12	HA	Covance, Harrogate, UK	mouse, monoclonal	1:1000 in IF
AlexaFluor 488	mouse IgG	Invitrogen, Paisley, UK	donkey, polyclonal	1:500 in IF

Table 4 Antibodies

2.1.7. General plasmids

Plasmid	Notes	Origin
p8.91	NL4.3 gag pol, tat and rev expression	[454]
p8.91Ex	NL4.3 gag pol, tat and rev expression modified	[470]
CSGW	HIV-1 vector encoding GFP	[455]
pMD.G	VSV-G expression plasmid	[471]
CNCG	MLV vector encoding GFP	[294]
CNCR	CNCG derived, encoding dsRed	[294]
CMVintron	MoMLV gag pol expression	[286]
HIV-2pack	HIV-2 gag pol expression	[456]
HIV-2GFP	HIV-2 vector encoding GFP	[456]
FP93	FIV gag pol expression	[457]
pGinSin	FIV vector encoding GFP	[457]
EIAV pony	EIAV gag pol expression	[461]
EIAV ponyGFP	EIAV vector encoding GFP	[472]
SIV3+	SIV gag pol expression	[458]
SIVGFP	SIV vector encoding GFP	[458]
pCIG3 N	N-tropic MLV gag pol expression	[286]
pCIG3 B	B-tropic MLV gag pol expression	[286]
huCTCR	CXCR encoding human TRIM5α	[185]
huCTCRΔE	huCTCR derived, single *EcoR*I site	made by Z. Keckesova
huCTCRδ	CXCR encoding human TRIM5δ	[293]
huCTCRγ	CXCR encoding human TRIM5γ	made by Z. Keckesova
rhCTCR	CXCR encoding rhesusTRIM5α	[433]
EXN	LNCX2 derived vector with HA tag	[342]
LNCX2TCyp	N-terminal tagged owl monkey TRIMCyp	[342]
EXNM7TC	N-terminal tagged rhesusTRIMCyp (E486K)	[186]
CFCRΔE	CXCR encoding Fv1N	[293]
EXNT21	MLV vector expressing HA tagged hu TRIM21	[342]
EXNT34	MLV vector expressing HA tagged hu TRIM34	[342]
pSRQ	MLV vector to clone short hairpin RNAs	Clontech, Mountain View, USA
pMT107	Plasmid encoding 6HIS tagged ubiquitin	[473]
pGEM-Teasy	Cloning vector with TA overhangs for Taq cloning	Promega, Southampton, UK
ZeroBlunt pCR4-TOPO	Cloning vector with blunt ends and topoisomerase	Invitrogen, Paisley, UK

Table 5 General plasmids

2.1.8. Generated plasmids

Name	Template	Primers (PCR/*OPCR*)	Enzymes	Target/ *SDM*
TOPO_T5UP1	SIRC T5 5'RACE	TS12 (RT), TS10 (RACE), TS8 (nested PCR)	-	ZeroBlunt pCR4-TOPO
TOPO_S6.2	SIRC T5 3'RACE	OligodT anchor (RT), TS2 (RACE), TS6 (nested PCR)	-	ZeroBlunt pCR4-TOPO
TOPO_2.1	cDNA SIRC	TS20, TS22	-	ZeroBlunt pCR4-TOPO
CTCRrbT5 (c2.1.1)	TOPO_2.1	-	*EcoR*I, *Csp45*I	CTCR
EXNrbT5	CTCRrbT5	TS35, TS36	*EcoR*I, *Not*I	EXN
EXNomtcΔ93 (c2B1)	LNCX2TCyp	GT472, GT474	*EcoR*I, *Not*I	EXN
EXNomtcΔ128 (c3B2)	LNCX2TCyp	GT473, GT474	*EcoR*I, *Not*I	EXN
EXNrhT5 (c4.2)	rhCTCR	GT437, GT273	*EcoR*I, *Not*I	EXN
EXNrhΔ59 (c1.3)	rhCTCR	GT471, GT273	*EcoR*I, *Not*I	EXN
CTCRrhΔ59	EXNrhΔ59	GT270, TS143	*EcoRI, Csp45*I	CTCRΔE
EXNrhΔ94 (c3)	EXNrhT5	TS90, GT273	*EcoR*I, *Not*I	EXN
CTCRrhΔ94 (c17)	EXNrhΔ94	TS77, GT270	*EcoRI, Csp45*I	CTCRΔE
EXNrhΔ129 (c5)	EXNrhT5	TS91, GT273	*EcoR*I, *Not*I	EXN
CTCRrhΔ129 (c21)	EXNrhΔ129	TS159, GT270	*EcoRI, Csp45*I	CTCRΔE
EXNhuT5 R332P (cR17)	R/ huCTCR	TS58, TS24	*MfeI, Not*I	EXN
EXNhuΔ58 (c1)	huCTCRΔE	TS84, GT273	*EcoR*I, *Not*I	EXN
CTCRhuΔ58 (c13)	EXNhuΔ58	TS158, GT270	*EcoRI, Csp45*I	CTCRΔE
EXNhuΔ92 (c1A1)	EXNhuT5	TS90, GT273	*EcoR*I, *Not*I	EXN
EXNhuΔ127 (c1B1)	EXNhuT5	TS93, GT273	*EcoR*I, *Not*I	EXN
EXNrhTC	EXNM7TC	SWJ35,GT487	*EcoR*I, *Not*I	EXN
EXNrhTCΔ94 (c1C1)	EXNrhTC	TS90, GT487	*EcoR*I, *Not*I	EXN
EXNrhTCΔ129 (c1D1)	EXNrhTC	TS91, GT487	*EcoR*I, *Not*I	EXN
p8.91ExHIV(S/HCA)	S/HCA [474]	TY2, TY3	*Bgl*II, *Not*I	p8.91Ex
p8.91ExHIV(H/SCA)	H/SCA [474]	TY2, TY3	*Bgl*II, *Not*I	p8.91Ex
EXNomtcNotI (c1)	EXNomtcΔ128	TS183, TS184	*Not*I, *Sal*I	EXNomtcΔCyp
EXNomtHuCypA (cH6)	Hela cDNA	TS141, TS142	*Not*I, *Sal*I	EXNomtcΔCyp
EXNomtHuCypB (c5)	TE671 cDNA	TS131N, TS132	*Not*I, *Sal*I	EXNomtcΔCyp
EXNomtHuCypC (c6)	TE671 cDNA	TS133N, TS134	*Not*I, *Sal*I	EXNomtcΔCyp
EXNomtHuCypE (c2n11)	TE671 cDNA	TS135N, TS136	*Not*I, *Sal*I	EXNomtcΔCyp
EXNomtHuCypF (cn9)	TE671 cDNA	TS137N, TS138	*Not*I, *Sal*I	EXNomtcΔCyp

EXNomtNup358Cyp	TE671 cDNA	TS129N, TS130	*Not*I, *Sal*I	EXNomtcΔCyp
EXNomtRbCyp18	pOPTHareCyp	TS141, TS208	*Not*I, *Xho*I	EXNomtcΔCyp
EXNomtcΔCyp (c2)	LNCX2TCyp	TS127, TS128	*EcoR*I, *Not*I	EXN
EXNFv1N	CFCRΔE	TS33, TS34	*EcoR*I, *Not*I	EXN
EXNFv1Cyp (c1.3)	CFCR432Cyp	TS85, TS33	*EcoR*I, *Not*I	EXN
EXNRBFv1N (cF34)	EXNrhT5	TS86, GT437	*EcoR*I, *EcoR*I	EXNFv1N
EXNRBFv1Cyp (cA4)	EXNFv1Cyp	-	*Xho*I, *Not*I	EXNRBFv1N
EXNBFv1Cyp (c15)	EXNRBFv1Cyp	TS85, TS185n	*Mfe*I, *Not*I	EXN
EXNT34R5 (cII.4)	EXNT34, huCTCR	*TS56+TS45, TS44+GT273*	*Mfe*I, *Not*I	EXN
EXNT34R5 R332P	EXNhuT5 R332P	-	*Hind*III	EXNT34R5
CFCR358*Mlu*I	CFCRΔE	TS29, TS30	-	*SDM* (CFCRΔE)
CFCR432*Mlu*I	CFCRΔE	TS31, TS32	-	*SDM* (CFCRΔE)
CFCR358Cyp	LNCX2TCyp (OMK)	TS27, TS28	*Mlu*I, *Csp45*I	CFCR358*Mlu*I
CFCR432Cyp	LNCX2TCyp (OMK)	TS27, TS28	*Mlu*I, *Csp45*I	CFCR432*Mlu*I
EXNT21aa284Cyp	EXNT21	TS154, TS43	*EcoR*I, *Not*I	EXNomtHuCyp A
EXNT21aa290Cyp	EXNT21	TS155, TS43	*EcoR*I, *Not*I	EXNomtHuCyp A
EXNT21aa309Cyp	EXNT21	TS156, TS43	*EcoR*I, *Not*I	EXNomtHuCyp A
pSRQ_rbT5_sh1	Primer annealing	GT389, GT390	*BamH*I, *EcoR*I	pSRQ
pSRQ_rbT5_sh2	Primer annealing	GT391, GT392	*BamH*I, *EcoR*I	pSRQ
pSRQ_rbT5_sh4.1	Primer annealing	TS65, TS66	*BamH*I, *EcoR*I	pSRQ
pSRQ_rbT6_sh6.3	Primer annealing	TS69, TS70	*BamH*I, *EcoR*I	pSRQ
pGEM-2LTR1	total DNA TE671	MH535, MH536	-	pGEM-Teasy

Table 6 Generated plasmids/ cloning strategy

OPCR, overlapping PCR; SDM, site directed mutagenesis

2.2. Methods

2.2.1. Isolation of DNA

The protocols used for the preparation of plasmid-DNA from bacteria are based on the principle of alkaline lysis [475]. To grow bacterial over night cultures for Miniprep DNA isolation, 2 ml of LB media containing selection drug were inoculated with bacteria picked from a single colony grown on an agar plate. The inoculated cultures were incubated overnight at 37°C while shaking at 250 rpm. Small-scale preparations of plasmid DNA were performed using the QIAprep Spin Miniprep Kit (Qiagen, Crawley, UK) or the PureLink Plasmid Miniprep kit (Invitrogen, Paisley, UK), following the protocols provided with the kits. Plasmids were usually eluted from the column by adding 50 µl of water. Depending on the plasmid, usually 4 to 10 µg of low copy plasmid DNA were isolated. Medium scale preparation of plasmid DNA was performed using the QIAfilter Plasmid Midi Kit (Qiagen, Crawley, UK) according to the manufacturer's protocol.

2.2.2. DNA extraction from agarose gels

Extraction and purification of DNA bands from agarose gels was performed using the QIAquick gel extraction Kit (Qiagen, Crawley, UK) according to the manufacturer's protocol. DNA fragments were eluted from the column by adding 30 – 50 µl of water.

2.2.3. Ligation of DNA-fragments

For ligation usually 2-4 µg plasmid DNA was digested with appropriate enzymes and vector or insert DNA fragments were gel purified and eluted into 50 µl of water. Concentrations for purified vector and insert DNA were ~ 0.04 to 0.08 µg/ µl. Concentrations of PCR products were measured using a NanoDrop spectrophotometer ND-1000 (Thermo Fisher Scientific Inc., Wilmington, USA) and had similar ranges. Usually 1 µl of digested and purified vector DNA was mixed with 3 µl of insert DNA or PCR product. Vector DNA sizes ranged between 3 kb and 10 kb, and insert DNA sizes between 500 bp and 2 kb. The molecule numbers were calculated using the formula: 1 pmol of 1000 bp dsDNA ≈ 0.66 µg. Using this equation the ligation mixes contained

~ 0.01 - 0.04 pmol of digested and gel purified vector DNA and ~ 0.05 - 0.2 pmol of insert DNA. The recommended molecule ratio of vector to insert is one to three. Vector and insert DNA were mixed with 2 µl 10 × ligase buffer, 1 µl (3 U) T4-DNA-ligase (Promega, Southampton, UK) and water to a final volume of 20 µl. After incubation at 25 °C for 2 h or at 4 °C overnight 10 µl of the ligation-mix were added directly to 80 µl competent bacteria for transformation. To avoid a high number of background colonies in the case of the DNA ends being compatible for religation, linearised vector-DNA was incubated for 30 min at 37 °C in a 1 × reaction buffer containing 1 U calf intestinal alkaline phosphatase CIP (Promega, Southampton, UK) and gel purified before ligation.

2.2.4. Preparation of chemical competent E.coli

To generate E.coli competent for heat shock transformation the following protocol was used. Hb101 E.coli were spread on agar plates without antibiotics and grown overnight at 37 °C. The next day 10 ml LB media were inoculated with a single Hb101 colony and bacteria were grown overnight shaking at 350 rpm at 37 °C. The next day 50 ml of LB media were inoculated with 1.25 ml of overnight bacterial culture. Bacteria were grown while shaking at 37 °C until an optical density of OD_{550nm}=0.45-0.55 was reached. The suspension was incubated on ice for 10 min. Bacteria were pelleted by centrifugation at 3000 rpm and 4 °C for 15 min. Pelleted bacteria were resuspended in 20 ml of TfB1 and left on ice for 5 min before they were centrifuged again under the same conditions as above. The bacterial pellet was resuspended in 2 ml of TfB2 and left again on ice for 10 min. Aliquots of 100 µl were pipetted into 0.5 ml Eppendorf tubes, which were immediately thrown into liquid nitrogen to snap-freeze competent cells. Transformation competent cells were stored at -80 °C and could be used for over 3 months without losing efficiency.

2.2.5. Transformation of E. coli by heat shock

In most cases, chemical competent Hb101 were used for transformation. 80 µl bacteria suspension was incubated with plasmid DNA or ligation reaction for 45 min on ice. After heat shock at 42 °C for 90 seconds the cells were directly plated on solid LB-plates containing ampicillin. In the case of kanamycin resistance, transformed cells were mixed with 800 µl of LB-medium and incubated for a further 30 min at 37 °C

while shaking. After centrifugation for 2 min at 6000 rpm, supernatants were removed and cells resuspended in 50 µl of LB medium. After plating the cells on LB-agar plates with antibiotic, colonies were grown overnight at 37 °C.

2.2.6. Analysis of DNA with restriction enzymes

Restriction digests of DNA were performed with enzymes from the manufacturers NEB (Hitchin, UK) and Promega (Southampton, UK). Enzymes and buffers were used as recommended by the manufacturer and reaction conditions were set according to their protocols.

2.2.7. Polymerase Chain Reaction (PCR)

PCR is a method to amplify a defined DNA sequence from a complex template DNA selectively. The flanking sequences of the target DNA are used to generate a sense and an anti-sense oligonucleotide primer (usually 18-30 bp). The primers are used as the starting point by the polymerase for amplification. Chain elongation in 5´ to 3´direction is achieved by addition of dNTPs. The steps of PCR amplification are as follows I) heat denaturation of the double-stranded template DNA at 95 °C, II) primer annealing to the complementary sequences of the single stranded target (45-60 °C), III) extension by the DNA polymerase at 68-72 °C. After primer extension the mixture is heated again to separate the strands. Cooling down the mixture allows the primers to hybridize with the complementary regions of newly synthesized DNA. Each cycle literally doubles the amplicon.

The reaction mixture was prepared on ice. Each reaction contained 250 µM dNTPs (each dNTP), 1 × polymerase buffer, 10 pmols of each primer, 100 ng to 1 µg template DNA and 1 µl (3 U) of PFU polymerase (Promega, Southampton, UK) made up with H_2O to a final reaction volume of 50 µl. Annealing temperature as well as elongation time were set depending on the primer composition and the length of the amplified sequence, respectively. Usually 34 cycles were performed. All nucleotide sequences of PCR products were confirmed by sequencing (Lark Technologies Incorporated, Essex, UK).

2.2.8. PCR-based mutagenesis

To generate plasmids carrying point mutations, deletions or insertions, PCR based mutagenesis was performed. To engineer point mutations into a plasmid, PCR was performed as described above using two primers with opposite orientation covering the desired point mutation. Usually, 34 cycles were performed with an elongation time of 15-25 min for each step, depending on the size of the plasmid into which the mutation was introduced. Primer annealing temperatures were between 53-55 °C. To avoid errors, PFU Turbo (Stratagene, La Jolla, USA) was used according to the protocol manufacturer. After the amplification, the template plasmid was digested by adding 2 µl of *Dpn*I directly to the PCR reaction mix and incubation at 37 °C for 2 h. After digestion, 10 µl of the mix were transformed into competent bacteria.

To create fusion proteins of TRIM5α and TRIM34 two overlap PCR reactions were performed. In the first reaction the reverse primer contained a 5' overhang complementary to the 5' region of the PCR product from the second reaction. The forward primer of the second PCR reaction had a 5' overhang complementary to the 3' end of the first PCR product. After 20 amplification rounds 1 µl of each PCR reaction were mixed and another 20 amplification rounds were performed including 0.2 pmol forward primer of the first PCR reaction and 0.2 pmol reverse primer of the second PCR reaction to generate the full-length overlap PCR product.

2.2.9. TaqMan qPCR

To measure viral DNA, Taqman quantitative PCR (qPCR) was performed using primer/probe sets specific to *GFP*, a late reverse transcriptase product (*LRT-P*) or viral *2-LTR* DNA circles (see 2.1.5.). Cells were infected in six-well plates in triplicate for 6 h (*GFP*), 12 h or 18 h (*LRT-P* and *2-LTR* DNA circles) with equal doses of DNase treated virus (final DNase concentration 70 U/ml for 2 h; Promega, Southampton, UK). After the incubation period total DNA was extracted from two samples using the QiaAmp DNA extraction kit (Qiagen, Crawley, UK). To enumerate infected cells, the third sample was subjected to FACS analysis 48 h after infection. As a negative control for plasmid contamination cells were incubated with viral supernatant that had been boiled for 5 min at 95 °C. Extracted total DNA (100-500 ng) was subjected to Taqman qPCR using the protocol described in [113]. Briefly, 5 µl of diluted plasmid standard or

total DNA sample were added to 20 µl PCR master mix containing 1 µl of each primer (300 nM final concentration), 0.5 µl of the probe primer (150 nM final concentration), 5 µl H_20 and 12.5 µl of 2 × QuantiTectProbe master mix (Qiagen, Crawley, UK) or 2 × Platinum qPCR Supermix (Invitrogen, Paisley, UK). In every experiment, a standard curve of the respective amplicon was measured in duplicate ranging from 10^1 to 10^5 copies in addition to a no-template control. To measure *GFP* the plasmid CNCG served as standard, for *LRT-P* the plasmid CSGW and for *2-LTR* circles the plasmid pGEM-2LTR1 or 2-LTR-LA [476] (see 2.1.5.) were used, and all dilutions were performed in an equal amount of salmon sperm carrier DNA in water (100 µg/ ml). The PCR cycling program for the *GFP* and the *LRT-P* amplicon consisted of initial incubations at 50 °C for 2 min and 95 °C for 10 min, after which 40 cycles of amplification were carried out at 15 s at 95 °C followed by 1 min at 60 °C. For 2-LTR circles usually 50 cycles were performed with 15 s at 95 °C followed by 90 s at 60 °C with two different primer/ probes and standard sets from [113] and [476]. For detection the ABI Prism 7000 sequence detection system was used (PE-Applied Biosystems, Foster City, USA). To analyse the experiments, usually the number of copies of amplicon per 100 ng total DNA was calculated and plotted.

2.2.10. Cloning of shRNAs into retroviral vector pSIREN RetroQ

Suitable silencing RNA target sequences were identified using the Clontech website http://bioinfo.clontech.com/rnaidesigner/sirnaSequenceDesignInit.do. Oligonucleotide sequences to generate short hairpin RNAs were designed using the Clontech website http://bioinfo.clontech.com/rnaidesigner/oligoDesigner.do, and ordered from Sigma Aldrich (Dorset, UK). Overlapping oligonucleotides encoding the shRNA were annealed using the following protocol. 10 µg of each oligonucleotide (1 µg/ µl) were mixed with 2.5 µl of 2 M NaCl solution in a total volume of 50 µl. The mix was boiled for 5 min at 98°C in a PCR block and subsequently cooled down at a rate of 0.1 °C per second. After annealing 350 µl H_2O, 40 µl 3 M NaAc and 1100 µl 100 % ethanol were added and the solution was briefly vortexed and then placed at -80 °C and incubated over night. To pellet the annealed oligos the solution was spun at 14000 rpm in a bench top centrifuge at 4 °C for 20 min. The DNA pellet was air dried and resuspended in 50 µl H_2O. The annealed oligos with overhanging single strand ends were then cloned

into the *EcoR*I and *BamH*I sites in the pSIREN RetroQ vector (Clontech, Mountain View, USA).

2.2.11. Cloning of rabbit TRIM5 by rapid amplification of cDNA ends (RACE)

Total RNA from SIRC cells was isolated using TRIzol (Invitrogen, Paisley, UK) according to the manufacturer's protocol. Briefly, 3×10^6 SIRC cells were taken up in 1 ml TRIzol and stored at –80 °C until further use. After thawing the lysate was incubated at 70 °C for 10 min followed by incubation at room temperature for 3 min. After addition of 0.2 ml chloroform and vigorous vortexing the mix was left for 3 min at room temperature before centrifugation at 13000 rpm, 4 °C for 15 min in a bench top centrifuge. The upper phase (~ 0.6 ml) was carefully transferred to a clean 1.5 ml Eppendorf tube, 0.5 ml of chloroform was added and the suspension was vigorously vortexed. After 3 min incubation at room temperature and centrifugation as before the top phase was again carefully transferred to a clean Eppendorf tube. To precipitate nucleic acids 0.5 ml of isopropanol were added and after vigorous shaking the suspension was incubated at room temperature for 10 min before centrifugation at 13000 rpm and 4 °C for 10 min in a bench top centrifuge. The isopropanol pellet was washed once with 1 ml of 75% ethanol and spun at 8000 rpm and 4 °C for 5 min. The pellet was air dried, dissolved in 170 µl H_2O and incubated at 55 °C for 10 min. To digest precipitated DNA, 20 µl DNAse buffer and 10 µl DNAse (1U/ µl, Promega, Southampton, UK) were added to the mix, which was incubated for 1 h at 37 °C. After that incubation period 20 µl termination mix (Promega, Southampton, UK) was added and the mix was vortexed. To precipitate the RNA, one starting volume (200 µl) of phenol:chloroform:isoamylalcohol (25:24:1) (Sigma Aldrich, Dorset, UK) was added, the mix was vortexed and spun at 13000 rpm and room temperature for 10 min. The top phase was transferred to a new Eppendorf tube and the extraction was repeated. After centrifugation as before, the top phase was transferred to a new Eppendorf tube and one volume of chloroform was added. The mix was vortexed vigorously and left at room temperature for 3 min before centrifugation at 13000 rpm and room temperature for 10 min. The upper phase (~ 225 µl) was transferred to a new Eppendorf tube and 50 µl of 7.5 M ammonium acetate solution was added. After addition of 2.5 volumes (~ 686 µl) of ice-cold 95 % ethanol and vigorous vortexing the suspensions was placed at -20 °C for 1-2 h to precipitate the RNA. The mix was spun in a bench top centrifuge at 13000

rpm and 4 °C for 30 min and the supernatant was discarded. After washing the pellet with 200 µl of ice-cold 80% ethanol the mix was spun again at 13000 rpm and 4 °C for 10 min. The pellet was air dried and resuspended in 80 µl H_2O DEPC. The concentration was determined using a NanoDrop spectrophotometer ND-1000 (Thermo Fisher Scientific Inc., Wilmington, USA) and was usually ~2-4 µg/µl.

Total RNA was used to generate cDNA using Amv reverse transcriptase (Promega, Southampton, UK). Before reverse transcription, 2 µl of total RNA isolated from SIRC cells were mixed with 5 µl 10 × Amv RT reaction buffer, 2.5 µl dNTP (10 mM), 5 µl dTT (0.1 M), 1 µl oligo dT primer (10 µM), 1 µl random primer (10 µM) and 31 µl H_2O. The mix was incubated for 2 min at 42 °C before adding 2.5 µl of Amv RT polymerase. The reaction mix was incubated for 48 min at 42 °C, before inactivation of the Amv RT enzyme by incubation at 72 °C for 15 min. The generated cDNA was used as a template for PCR amplification with primers TS1 and TS16 (see 2.1.4.). PCR products were cloned into a ZeroBlunt TOPO cloning vector (Invitrogen, Paisley, UK) and sequenced. The partial rabbit TRIM5 cDNA sequence cloned into ZeroBlunt TOPO was used to design internal primers.

To amplify the cDNA ends of rabbit TRIM5 a second-generation 5'/3' RACE kit (Roche Applied Science, Burgess Hill, UK) was used according to the manufacturer's instructions. To generate total cDNA, isolated total RNA from SIRC cells was applied to reverse transcription using the RT enzyme provided with the kit and primers TS12 (5'RACE) or oligo dT-anchor primer (3'RACE). Briefly, 4 µl of 5 × RT buffer (vial 1) were mixed with 2 µl dNTP (vial 3), 1 µl primer (12.5 µM) (TS12 or oligo dT-anchor primer (vial 8)), 1 µl total RNA from SIRC cells (2 µg/ µl), 1 µl RT enzyme (vial 2) and 11 µl H_20. The reaction mix was incubated at 55 °C for 60 min and then placed at 85 °C for 5 min. The RT products were purified using High Pure PCR purification kit (Roche Applied Science, Burgess Hill, UK). For purification 20 µl of RT product was mixed with 100 µl of DNA binding buffer and applied to a purification column provided with the kit. The column was spun at 8000 g for 30 s and the flow through was discarded. The column was washed by adding 500 µl washing buffer and spun as before. The flow through was discarded and the column was washed again by adding 200 µl washing buffer. After 2 min centrifugation at 13000 g the PCR product was eluted from the column into a clean 1.5 ml Eppendorf tube by adding 50 µl elution buffer and centrifugation for 30 s at 8000 g. For the 3'RACE reaction 1 µl of purified cDNA was mixed with 1 µl anchor primer (vial 9), 1 µl TS2 (12.5 µM), 1 µl dNTP (vial 3), 5 µl

10 × PFU buffer, 0.5 µl PFU Turbo (Stratagene, La Jolla, USA) and 38.5 µl of H_2O. Thirty-four cycles were performed with a primer annealing temperature of 55 °C and 1 min PFU elongation time at 68 °C. The nested PCR was performed by adding 1 µl TS6 (12.5 µM) and 1 µl anchor primer (vial 9) with the same PCR conditions as described above. Finally, 4 µl of the 3'RACE nested PCR product was used for direct cloning into the ZeroBlunt TOPO cloning vector (Invitrogen, Paisley, UK). The correct 3' end was found in plasmid TOPO_S6.2.

To tail the generated cDNA with polyA at the 3' end, 19 µl of purified cDNA were mixed with 2.5 µl 10 × terminal transferase (TT) buffer (vial 5) and 2.5 µl ATP (2 mM) (vial 4) and incubated at 94 °C for 3 min. After incubation on ice for 1 min 1 µl TT (vial 6) was added and the reaction was placed at 37 °C for 25 min, 70 °C for 10 min and then left on ice. For the 5' RACE PCR 5 µl of polyA tailed cDNA was mixed with 1 µl oligo dT-anchor primer (vial 8), 1 µl TS10 (12.5 µM), 1 µl dNTP (vial 3), 5 µl 10 × PFU buffer, 0.5 µl PFU Turbo (Stratagene, La Jolla, USA) and 36.5 µl of H_2O. The PCR was performed using the same conditions as for the 3'RACE and the nested PCR was performed using 1 µl of primer TS8 (12.5 µM) and 1 µl anchor primer (vial 9), as before. The correct 5' rabbit TRIM5 sequence was found in plasmid TOPO_T5UP1.

Subsequently, primers TS20 and TS22 designed against the correct 5' and 3' ends respectively were used to amplify the entire rabbit TRIM5 cDNA from SIRC total cDNA, which was cloned into ZeroBlunt TOPO cloning vector resulting in the plasmid TOPO_2.1. The obtained sequence was used in BLAST [477] against the known rabbit genome and a genomic contig (GenBank accession no. AC186253) was identified covering the entire rabbit TRIM5 gene. In addition, it also revealed another rabbit TRIM gene, subsequently identified as rabbit TRIM6, which was assembled by successive alignment of rabbit TRIM5 exons. The identified rabbit TRIM5 cDNA sequence has the GenBank accession number EU014879.

2.2.12. Evolutionary analysis by generation of phylogenetic tree

The nucleotide sequences for human TRIM genes 22 (RefSeq no. NM_006074), 34 (RefSeq no. NM_021616), 5_ (RefSeq no. NM_033034), and 6 (RefSeq no. NM_058166); porcine TRIM5 (GenBank no. AY970971); African green monkey TRIM5 (GenBank no. AB210050); rhesus macaque TRIM5 (GenBank no. AY523632);

gorilla TRIM5 (AY923178); murine TRIM genes 6 (RefSeq no. NM_001013616), 5 (RefSeq no. NM_175677), 12 (RefSeq no. NM_023835), 30 (RefSeq no. NM_009099), 34 (GenBank no. AF220139), and 21 (GenBank no. CT010336); bovine TRIM genes 5 (Lv1) (GenBank no. DQ380509), 5b (GenBank no. AY970972), and 5d (RefSeq no. XM_864679); and rabbit TRIM5 (GenBank no. EU014879) and TRIM6 were manually aligned using the application Se-Al [478]. A maximum-likelihood phylogenetic tree was then reconstructed under the general time-reversible model of nucleotide substitution with a proportion of invariable sites and gamma-distributed rate heterogeneity, using the program PAUP* [479]. The robustness of the tree topology was assessed by bootstrap analysis with 1,000 replicates. The phylogenetic tree was generated by Dr Stephane Hué (University College London, Division of Infection and Immunity, Department of Infection).

2.2.13. Analysis of rabbit and hare B30.2 domain polymorphisms

Genomic DNA from liver samples of two small wild rabbits caught in the UK (UKRb1, UKRb2) and four French farmed rabbits (Rb1, Rb2, Rb3, Rb4) were analysed for their TRIM5 B30.2 sequence. In addition, two rabbit cell lines, SIRC and EREP, were investigated. One ear sample and two liver samples from three different European brown hares were obtained and their genomic DNAs were analysed for the TRIM5 B30.2 coding region as described below. In addition, a hare kidney fibroblast cell line (kind gift from Jean Francois Vautherot) was examined. Rabbit and hare samples were obtained from an exotic meats butcher (McKenna meats). Total DNA was prepared using the QiaAmp DNA extraction kit (Qiagen, Crawley, UK) following the manufacturer's protocol. Primer TS82 complementary to an intronic sequence between exon 7 and 8 and primer TS83 complementary to a non-coding sequence downstream of exon 8 were designed according to the genomic contig of the rabbit TRIM5 locus and used to amplify exon 8 from rabbit and hare genomic DNA isolated from the animals or the cell lines above. Proof reading polymerase Pfu (Promega, Southampton, UK) was used to avoid PCR errors. Thirty-four PCR cycles were performed with a primer annealing temperature of 54 °C and an elongation time of 2 min at 72 °C. Gel purified PCR fragments were sequenced directly using primers TS82 and TS83 (Lark Technologies Incorporated, Cogenics, Essex, UK) and sequences were analysed using the software program DNADynamo (www.bluetractorsoftware.co.uk).

2.2.14. Virus preparation and infection assay

Infections and fluorescence activated cell sorting (FACS) were performed as described in earlier studies [185, 293, 480, 481]. Viruses were produced by transient transfection of 293T cells [467] with three plasmids using Fugene 6 (Roche Applied Science, Burgess Hill, UK) according to the manufacturer's protocol.

Briefly, for a ~80 % confluent 10 cm dish of 293Ts, 1.5 μg of vector DNA, 1 μg of GagPol encoding plasmid and 1 μg of VSV-G Env expression plasmid pMD.G (see 2.1.7.) were mixed in a total volume of 15 μl and added to a separate Eppendorf tube in which 200 μl OptiMem were pre-mixed with 10 μl of Fugene 6 (Roche Applied Science, Burgess Hill, UK). After 15 min incubation at room temperature and replacement of the 293T cell media with 8 ml fresh media (containing 10 % BS), the mix was added drop-wise onto the 293T cells. The viral GFP encoding vectors used, have been described in earlier studies (MLV-N and MLV-B [286], HIV-1 [454, 455], HIV-2[482], FIV [457], EIAV [472], SIVmac [458, 483]). The media was replaced 24 h post transfection with 8 ml fresh media and virus containing supernatant was harvested 48 and 72 h post transfection and filtered through a 0.45 μm filter before freezing at -80 °C. To determine infectious titres, 10^5 cells were plated in 6-well plates and infected the next day with 1 ml of viral supernatant diluted usually in a three-fold serial dilution series to obtain six different concentrations. Polybrene was included in the infection mix at a final concentration of 8 μg/ ml. Infected cells were enumerated 48 h post infection by measuring the GFP expression using FACS (BD Bioscience, Oxford, UK).

2.2.15. Generation of stable cell lines ectopically expressing proteins

Cell lines ectopically expressing proteins of interest were usually generated by infecting 10^5 cells in 6-well plates with 1 ml of neat viral supernatant from transfected 293T cells in the presence of 8 μg/ ml polybrene. Usually, the virus was spin inoculated onto the cells by centrifugation at 500 g for 1 h at 25 °C. In case of virus delivering a vector containing the neomycin resistance gene, cells were put under drug selection 48 h after transduction and kept in media containing 1 mg/ml G418 (Calbiochem, Merck Chemicals Ltd., Nottingham, UK) until untransduced control cells were dead, usually taking 1-2 weeks. In case of vectors with puromycin resistance, cells were grown in

media containing 1-7 µg/ µl puromycin depending on the cell line and selection normally took 1-3 days until control cells were dead. For single cell cloning cells were diluted to 1 cell per 500 µl media containing the selection drug and 500 µl was plated per well into 24-well plate dishes. Single cell clones were usually expanded first to 6-well plates and then to 10 cm plates before analysis.

Cell line	Drug	Concentration
SIRC	Puromycin	7 µg/ml
HareKF	Puromycin	10 µg/ml
CRFK	G-418	1 mg/ml
TE671	G-418	1 mg/ml
Hela	G-418	1 mg/ml

Table 7 Concentrations for drug selection of specific cell lines

2.2.16. Time of CSA addition experiment

To test for how long TRIMCyp or Fv1Cyp restricted infection can be rescued with CsA, 2×10^5 unmodified CRFK cells or CRFK cells expressing the restriction factor were incubated with a single dose of VSV-G pseudotyped, GFP encoding HIV-1 or MLV-B viral vectors at 4 °C for 1 h. The supernatant containing non-bound virus was removed and cells were washed using 2 ml cold media. After adding 1ml warm media to each well and 5 µM CsA to the 0 h time point, cells were incubated at 37 °C for 10 min to allow infection. To stop acidification of endosomes and thus VSV-G pseudotyped viral infection, ammonium chloride (NH_4Cl) was added to CRFK cells to a final concentration of 40 mM. The 10 min pulse of infection was chased by adding 5 µM CsA at different time points after NH_4Cl addition. As a control for NH_4Cl function the drug was added before the pulse. Usually, the media was changed the next day to media without CsA and NH_4Cl and infected cells were enumerated 48 -72 h post infection by FACS.

2.2.17. SDS-polyacrylamide-gel electrophoresis (SDS-PAGE)

A sodium dodecylsulfate-polyacrylamide gel can be used to separate proteins by their molecular weight under denaturing conditions. Laemmli gels were generated as

originally described [484] using an acrylamide concentration of normally 10 % for the separating gel and 6 % for the stacking gel. Briefly, for the separating gel 10 ml AcrylaFLOWGel (Flowgen Bioscience, Nottingham, UK), 4 ml of BisacrylFLOWGel (Flowgen Bioscience, Nottingham, UK), 150 µl of 20 % (w/v) SDS, 11.25 ml of 1 M Tris/HCl (pH 8.8), 4.5 ml water and 10 µl TEMED (Bio-Rad, Hemel Hempstead, UK) were mixed in a 50 ml Falcon Tube before starting gel polymerisation by adding 150 µl of 20 % ammonium-persulfate (APS) (Bio-Rad, Hemel Hempstead, UK). The separating gel was quickly poured between two glass plates of the Bio-Rad PAGE Gel casting system (Bio-Rad, Hemel Hempstead, UK). To avoid formation of air bubbles, 1 ml of isopropanol was carefully pipetted on top of the separating gel. After polymerisation the isopropanol was washed away with water and the excess water was removed by using Whatman paper strips (Bio-Rad, Hemel Hempstead, UK). To prepare the stacking gel, 2 ml of AcrylaFLOWGel, 0.8 ml of BisacrylFLOWGel, 50 µl of 20 % (w/v) SDS, 1.25 ml of 1 M Tris/HCl (pH 6.8), 5.87 ml water and 10 µl TEMED was mixed in a 50 ml Falcon tube before starting gel polymerisation by adding 50 µl APS. The solution was mixed and carefully added on top of the separating gel avoiding formation of air bubbles. The comb was inserted and the gels were left for 15 min at room temperature for gel polymerisation. The gels were wrapped in wet tissues and could be kept at 4°C for up to 1 week. Gels were run in SDS PAGE running buffer and PageRuler protein marker (Fermentas, York, UK) was used as a protein size reference.

2.2.18. Western blotting

Cells were usually lysed by addition of denaturing protein sample buffer. To shear the genomic DNA the samples were sonicated using the small tip with 8 pulses of 1 s each. The samples were boiled at 98 °C for 5 min. Aliquots of cell lysates were loaded onto SDS 10 % polyacrylamide gels for electrophoresis. Subsequently, the separated proteins were transferred to a polyvinylidene difluoride membrane (GE Healthcare, Little Chalfont, UK) usually using semiphor transfer buffer and a semidry blotter (Bio-Rad, Hemel Hempstead, UK). Blotting conditions were 1mA/ cm^2 for 30 min. Membranes were incubated for 1h in TBS containing 0.1 % Tween 20 (TBS-Tween 20) and 5 % (w/v) non-fat milk powder to saturate non-specific binding sites. The membrane was briefly washed in TBS-Tween 20. The primary antibody was diluted in TBS-Tween 20 containing 1 % (w/v) non-fat milk powder to a given concentration (Table 4) and the

membrane was incubated shaking for 1 h at room temperature. After being washed three times with TBS-Tween 20, the membrane was incubated with secondary antibody conjugated with peroxidase (GE Healthcare, Little Chalfont, UK) in TBS-Tween 20 containing 1 % (w/v) non-fat milk powder for 1 h shaking at room temperature and subsequently washed three times as described above. Membrane- bound secondary antibody was detected by chemiluminescence using ECL western blotting detection reagent (GE Healthcare, Little Chalfont, UK). Chemiluminescence was measured by autoradiography using Hyperfilm ECL (GE Healthcare, Little Chalfont, UK).

2.2.19. Purification of 6HIS-ubiquitinated proteins using Ni-NTA beads

To purify 6HIS tagged ubiquitinated proteins a protocol from the Tansey lab was followed. Briefly, 5×10^6 293T cells were plated in 10 cm plates and co-transfected the next day with 1.5 µg of pMT107 encoding for 6HIS tagged ubiquitin [473] and 1.5 µg EXN plasmids encoding different restriction factors or empty EXN vector as a control, made up in a total volume of 15 µl TE pH 8.0. For transfection 200 µl OptiMem was pre-mixed with 18 µl Fugene 6 (Roche Applied Science, Burgess Hill, UK) and the plasmid mix was added. Twenty-four hours after transfection the media was removed, 293T cells were washed in 10 ml PBS once, scraped in 1 ml ice cold PBS, transferred into a 1.5 ml Eppendorf tube and placed on ice. The cells were pelleted by 45 s centrifugation at 13000 rpm in a bench top centrifuge and the supernatant was discarded. Cell pellets were resuspended in 500 µl PBS by vortexing and 20 µl were transferred to a tube containing 50 µl of 2 × Laemmli sample buffer (input control). After mixing by pipetting up and down and boiling for 10 min the samples were frozen at -20 °C. The remaining cells were pelleted as before, taken up in 1 ml buffer A and mixed carefully by pipetting up and down. Cell lysates were sonicated using the small tip with 8 pulses of 1 s each. For each transfected 10 cm plate 50 µl of 50 % NiNTA resin were required. To equilibrate Ni-NTA resin 2 × 500 µl of 50 % Ni-NTA were pelleted in a microfuge for 7 s at 13000 rpm. The resin in each tube was washed 3 times in 1 ml of buffer A before resuspending both resins in 250 µl buffer A each and pooling the resins for a total volume of 1 ml. Fifty microliter of equilibrated Ni-NTA resin was added to each tube of cell lysate which were then rotated for 3 h at room temperature. After incubation cell lysates were spun for 10 s at 13000 rpm at room temperature and the supernatant was carefully removed leaving 100 µl behind. The Ni-NTA agarose

with bound proteins was then washed in 1 ml buffer A and pelleted as before. Washing in buffer A was repeated once before washing the resin twice in buffer A/TI always with pelleting steps as before in between the washing steps. After an additional wash with 1 ml of buffer TI the supernatant was removed leaving 100 µl behind. The sample was spun again as before. The supernatant was fully removed by plunging a 25G needle directly into the resin. The resin was resuspended in 100 µl of 2 × Laemmli buffer containing 0.3 M imidazole to elute the bound 6HIS-ubiquitinated proteins from the Ni-NTA resin and boiled for 10 min. Samples were stored at -20 °C before analysis by western blotting.

2.2.20. Immunofluorescence and confocal microscopy

Transduced CRFK cell lines were seeded on glass cover slips in 24-well cell culture plates at a concentration of 2.5×10^4 cells per well. When measuring sensitivity to proteasome inhibition the cells were treated the next day for 1-6 h with 8 µg/ml MG132. The cells were washed three times with warm 1 × PBS and fixed with 500 µl of a 4 % paraformaldehyde (w/v) solution for 10 min at room temperature. Subsequently, the cells were washed again three times with 1 × PBS and were then either directly used for further preparation or stored for up to 3 days at 4 °C. For permeabilisation the cells were incubated for 5 min with 500 µl of a 0.2 % triton-x-100/ PBS (v/v) solution and washed 3 times with warm 1 × PBS prior to incubation with the primary antibody. The primary antibody was diluted to the desired concentration in a 1 × PBS buffer containing 5 % bovine serum (v/v). After 45 min incubation at room temperature the cells were washed 3 times with 1 × PBS for 10 min and incubated with the secondary antibody conjugated with fluorescent dye Alexa 488, diluted 1/ 500 in a 1 × PBS buffer containing 5 % bovine serum (v/v). After 45 min incubation at room temperature in the dark, the cells were washed three times with warm 1 × PBS. Finally, the cells were washed with water and mounted on glass slides using Vecta-shield mounting media (Vector Laboratories, Burlingame, USA) containing DAPI (4',6 diamidino-2-phenylindole) to visualise cell nuclei and then sealed with nail polish. Immunofluorescence pictures were obtained using a Leica confocal microscope following protocols provided by the manufacturer. In general, pictures were obtained using the 63 × oil immersion objective.

3. Identification and characterisation of rabbit TRIM5

3.1. Introduction

The European rabbit (*Oryctolagus cuniculus*) together with the brown hare (*Lepus europaeus*) and members of 9 other genera belong to the *Leporidae* family of the order *Lagomorpha*. *Oryctolagus cuniculus* is distributed worldwide, mainly due to its extensive domestication and all breeds are classified in this genus. The phylogenetic relationships between individual genera of the *Leporidae* family are not fully understood but hares appear to have separated from a cluster of rabbits including the European rabbit ~11.8 million years ago (FIG 32). However, *Oryctolagus cuniculus* only diverged less than ~9.4 million years ago, suggesting that hares are evolutionary older than rabbits.

Rabbits are hosts for endogenous retroviruses [485] and have been the host for the rabbit endogenous lentivirus type K (RELIK) [486]. RELIK is the first endogenous lentivirus to be described. Moreover it is one of the oldest endogenous lentiviral genomes, that has been identified and the only one found in rabbits. More than 7 million years old, the rabbit endogenous lentivirus was suggested to be the common ancestor for all exogenous lentiviruses found today [486]. Interestingly, a RELIK homologue could also be identified in European brown hares [487]. The observation that hares share integration sites of RELIK with rabbits indicates that RELIK was present in the common ancestor of hares and rabbits suggesting that lentiviruses are at least 11.8 million years old. This indicates that lentiviruses are much older than previously thought.

Several studies have investigated the possibility of infecting rabbits with HIV-1 and their use as a small animal model for infection. Filice et al. described in Nature in 1988 the first attempted use of the domestic rabbit as a potential animal model for HIV-1 infection [488]. In this study rabbits were inoculated intraperitoneally with HIV-1 or infected H9 cells which caused apparent infection and a specific antibody response in two of three rabbits [488]. However, no disease symptoms could be observed. In a study prior to this, two rabbit T-cell lines and one rabbit macrophage line were infected with a high dose of HIV-1. RT enzymatic activity in the supernatants was detected with a peak 14 days after infection [489]. However, the two T-cell lines used in this study were transformed with herpes virus ateles and HTLV-I, respectively and the macrophage cell

line was transformed with SV40. In addition, untransformed peripheral blood lymphocytes (PBLs) isolated from rabbits did not support HIV-1 infection, strongly suggesting that the observed infection of the transformed cell lines does not reflect *in vivo* replication. Moreover, it was also observed that rabbits pre-infected with HTLV-I and subsequently infected with HIV-1 have an increased immune responses as well as disease symptoms like weight loss and diarrhoea [490]. Another study investigated the immune response to secondary infection in HIV-1 infected rabbits and demonstrated a diminution in the cellular immune response to *Mycobacterium bovis* [491]. A persistent immune response against HIV-1 proteins lasting for up to 3 years could be achieved when rabbits were inoculated multiple times with high doses of cell-free HIV-1 [492].

However, despite many attempts to use rabbits as an animal model for HIV-1 and despite promising early results, a sustained infection with pathological signs of immunodeficiency could not be achieved. The need for a high dose of virus inoculum to induce a serological response in rabbits indicates that rabbits are in general poorly permissive for HIV-1 infection.

In fact, *in vitro* studies of various rabbit cell lines demonstrated that they are poorly susceptible to infection by certain retroviruses including HIV-1, even when the specific receptor requirements are bypassed by pseudotyping the virus with the G-protein from Vesicular Stomatitis Virus (VSV-G) [297, 489, 493-495]. The reasons why rabbit cells are in general poorly permissive are not well understood, but could in theory be explained by 1) factors that are important for retroviral replication that are missing from rabbit cells and/or 2) negative acting factors that inhibit viral replication of specific retroviruses. Supporting the first theory one publication suggests that the rabbit cell line SIRC is poorly permissive for HIV-1 VSV-G pseudotyped vector infection due to the lack of a factor involved in pre-integration complex (PIC) trafficking [493]. In a study looking at the permissivity of a range of cell lines from different species to VSV-G pseudotyped viral vector infection it was demonstrated that HIV-1 GFP infects rabbit cells 10-100 fold less efficiently than SIVmac GFP [494]. Studying non-permissivity of diverse cell lines to retroviral infection revealed that TRIM5α proteins have a major role in restricting such infections [184, 185, 299, 496]. It was therefore investigated whether rabbits express an antiretroviral TRIM5 protein.

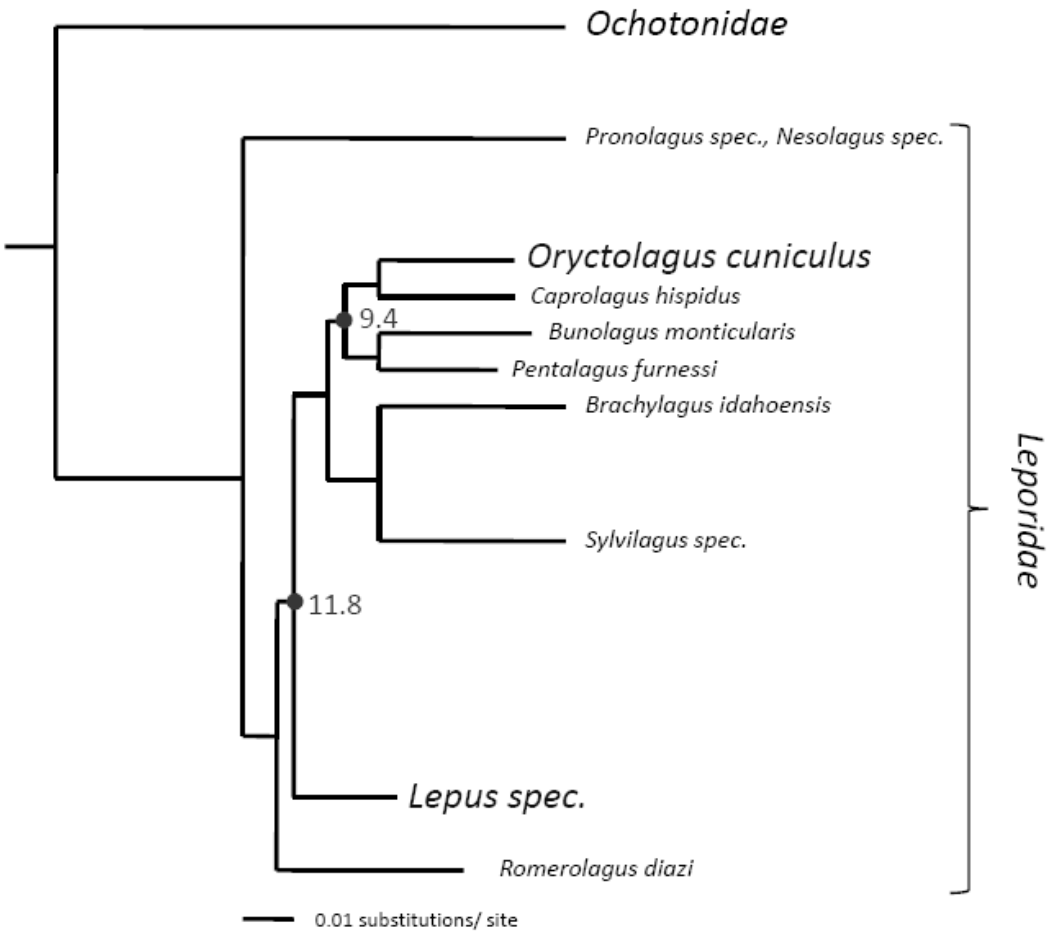


FIG 32 Phylogenetic tree of the order *Lagomorpha*

The phylogenetic tree shows the relationship between the European rabbit (*Oryctolagus cuniculus*) and the hare genus (*Lepus*). Numbers at branches indicate the estimated age in million years at which branches separated from each other. Hares separated approximately 12 million years ago from the ancient rabbit. The group including *Oryctolagus cuniculus* evolved approximately 9 million years ago. Figure adapted from [497].

3.2. Results

3.2.1. Rabbit cells strongly restrict HIV-1, HIV-2 and FIV infection

Rabbit cells have been demonstrated by several studies to be poorly susceptible to infection by certain retroviruses, even when the specific receptor requirements are bypassed by pseudotyping the virus with the G-protein from Vesicular Stomatitis Virus (VSV-G) [297, 493, 494]. VSV-G pseudotyped GFP encoding vectors of human immunodeficiency virus type 1 and 2 (HIV-1, HIV-2), feline immunodeficiency virus (FIV), equine infectious anaemia virus (EIAV), simian immunodeficiency virus from macaques (SIVmac) and N- and B- tropic murine leukaemia virus (MLV-N, MLV-B) were prepared (see 2.2.14.). These were then titrated on Crandell Reese feline kidney cells (CRFK) and infectious titres were compared to the rabbit corneal epithelial cell line (SIRC). Infectious titres of MLV-N, MLV-B, EIAV and SIVmac GFP viral vectors were only moderately reduced in SIRC cells compared to CRFK cells by 9 to 20-fold whereas infection by HIV-1, HIV-2 and FIV GFP vectors was strongly impaired in the rabbit cell line by 300 to 1000-fold (FIG 33A and B). For each virus identical stocks were used on CRFK and SIRC cells, suggesting that the change in infectious titer for HIV-1, HIV-2 and FIV is not reflected by differences in the virus dose.

3.2.2. HIV-1 capsid determines low infectivity in rabbit cells

The difference in permissivity to SIVmac and HIV-1 infection in rabbit cells was analysed more carefully with the aim of identifying the viral determinant for the low infectivity of HIV-1. To do this I used viruses with chimaeric Gag proteins, schematically depicted in FIG 33C. HIV-1 CA chimaeras HIV(S/HCA) and HIV(H/SCA) and SIV Gag chimaera SIV(HIVCA-p2) have been described in [474] and [498], respectively. HIV(H/SCA) and HIV(S/HCA) Gags were cloned from the original plasmids, gifts from Joe Sodroski, into p8.91Ex (see 2.1.8.). Infectious titres of VSV-G pseudotyped GFP viral vectors were determined on CRFK and SIRC cells and compared. HIV-1, HIV(S/HCA) and SIV(HIVCA-p2) viruses demonstrated 200 to 1000-fold lower infectious titres in SIRC cells compared to CRFK cells. However, replacement of the first 146 amino acids of HIV-1 CA with the sequence from SIVmac resulted in a virus that was only ~10-fold less infectious in SIRC cells compared to

CRFK cells, similar to the difference seen for SIVmac (FIG 33D and E). This result suggests that the determinant responsible for the restriction of HIV-1 infection in rabbit cells is located in the first 146 amino acids of CA.

3.2.3. Identification of rabbit TRIM5

The observation that the non-permissive phenotype of SIRC cells depends on the capsid of the virus suggests that a rabbit TRIM5 protein may be involved in restricting infection. To test whether rabbit cells express a TRIM5 homologue with antiretroviral activity in SIRC cells, forward and reverse primers were designed to TRIM5α regions that are conserved in several species and used to PCR amplify a TRIM5α fragment from cDNA prepared from SIRC total mRNA extracts. This sequence with high similarity to primate TRIM5α cDNAs was then used to design PCR primers for 5' and 3' rapid amplification of cDNA ends (RACE) (see 2.2.11). The full-length cDNA encoding for rabbit TRIM5α was amplified, sequenced and cloned into the retroviral expression vector CXCR described in previous studies [185, 293]. BLAST [477] searching the rabbit genome and successive identification of exon and intron sequences revealed a genomic organisation similar to that found in the TRIM5 genes of other species. Rabbit TRIM5 consists of 8 exons of which exon 8 encodes a PRY-SPRY domain (FIG 34). Alignment of the rabbit TRIM5 protein sequence to rhesus macaque and human TRIM5 shows that rabbit TRIM5 is similar in the RING and B-box domains whereas the coiled coil and PRY-SPRY domain differ significantly (FIG 35). The PRY-SPRY domain of rabbit TRIM5 differs mainly in the variable regions V1-V4 but is relatively well conserved in the beta sheets. It therefore most likely has a similar structure as the human or rhesus macaque TRIM5 B30.2 domains. Interestingly, rabbit TRIM5 has a longer V1 but a shortened V3 region compared to human and rhesus macaque TRIM5 suggesting differences in antiviral specificity.

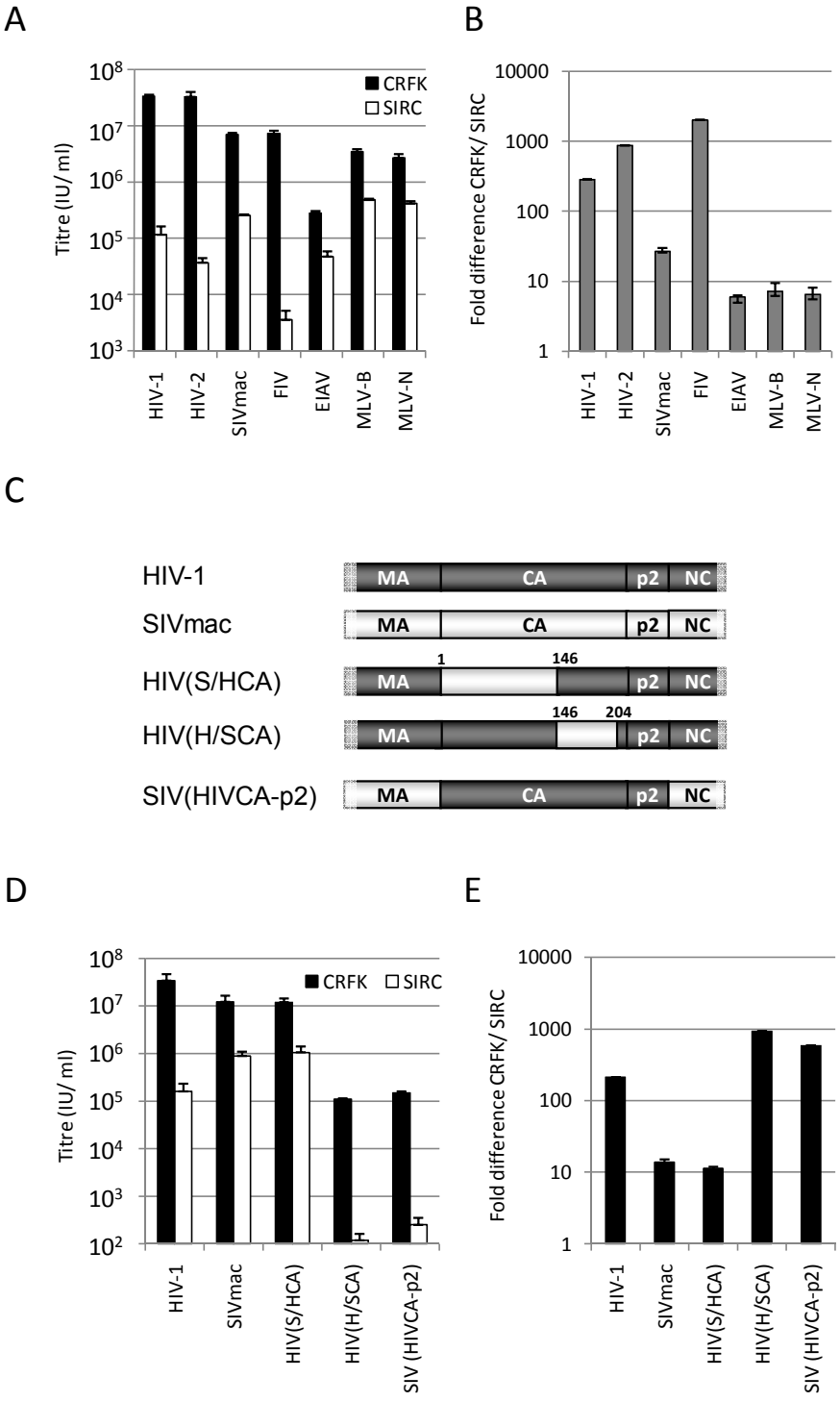


FIG 33 Comparison of permissivities of CRFK and SIRC cells to retroviral infection

A) Feline CRFK and rabbit SIRC cells were infected with the VSV-G pseudotyped GFP encoding retroviral vectors shown and infectious titres were calculated at three different virus doses. Error bars are standard deviations. B) Fold difference in infectious titres comparing CRFK cells and SIRC cells. C) Schematic presentation of SIVmac-HIV-1 chimaeric Gag proteins used to generate chimaeric viral GFP vectors. D) Infectious titres of SIVmac-HIV-1 chimaeric GFP vectors on CRFK and SIRC cells. Titres and error bars were calculated as in A). E) Fold differences in infectious titres of D). The data are representative of two independent experiments.

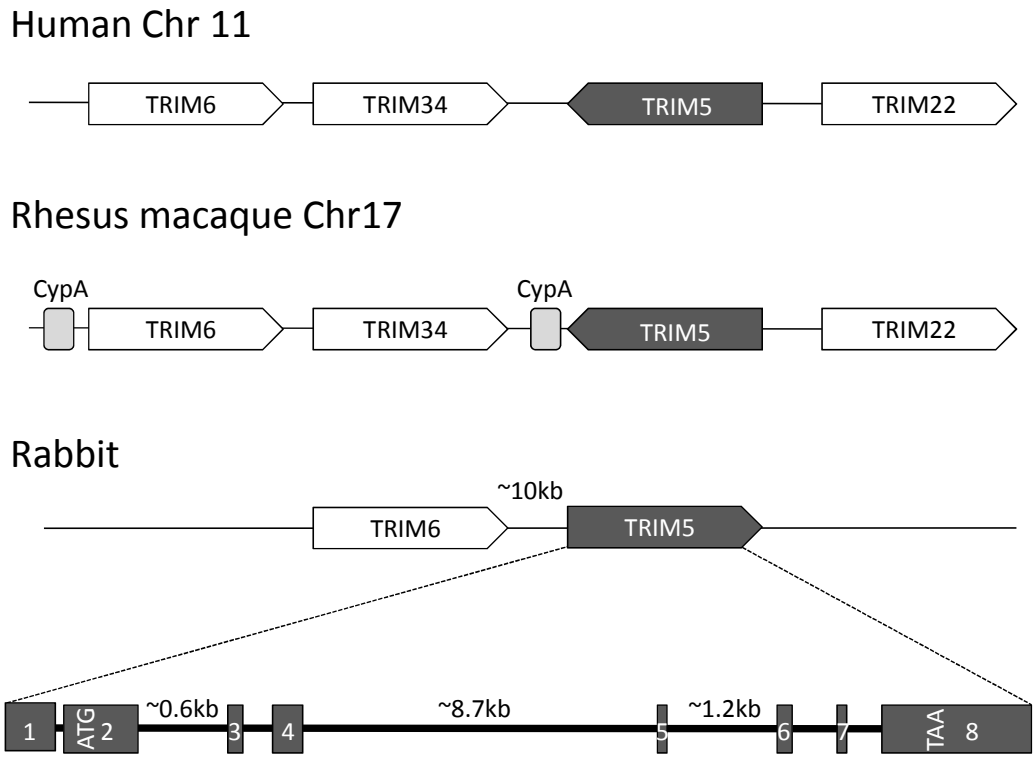


FIG 34 Genomic organisation of the TRIM5 gene loci in humans, rhesus macaques and rabbits

Depicted is a schematic comparison of the human TRIM5 gene locus on chromosome 11, rhesus macaque TRIM5 locus on chromosome 17 and the rabbit TRIM5 locus. Rabbit TRIM5 is located ~10kb upstream of the rabbit TRIM6 gene and consists of 8 exons of which exon 2-8 encode for the protein. In contrast to the rhesus macaque TRIM5 gene locus, no CypA insertions were found in proximity to the human and rabbit TRIM5 gene.

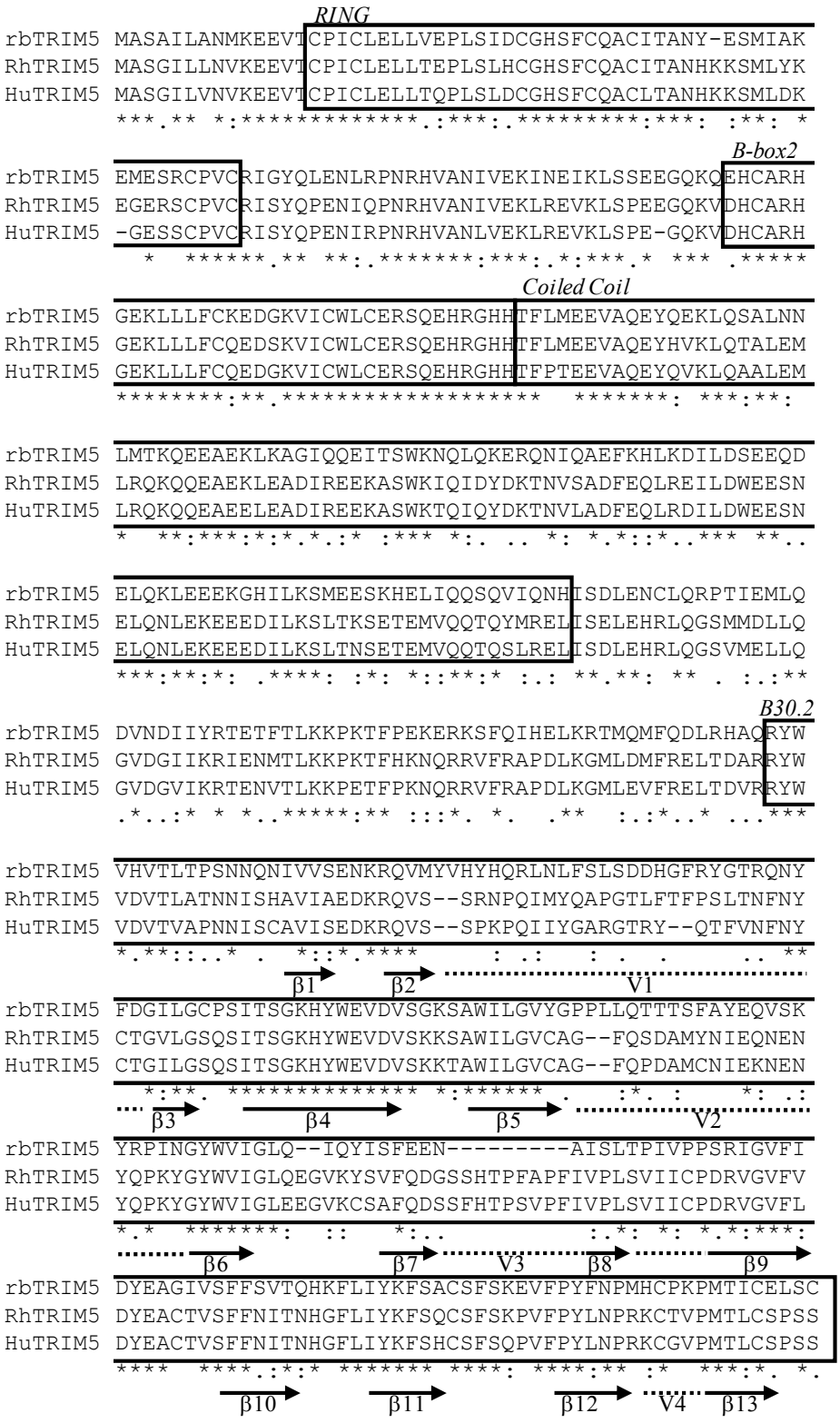


FIG 35 Amino acid alignment of human and rhesus macaque TRIM5α and rabbit TRIM5

Alignment of human (Hu), rhesus macaque (Rh) and rabbit (rb) TRIM5 proteins. Boxes indicate the regions of the RING, B-box (BB), coiled coil (CC) and B30.2 domain. Arrows indicate beta sheets β1 to β13 and dashed lines indicate variable regions V1-V4. Identical (*), highly similar (:) and weakly similar (.) amino acids are indicated.

3.2.4. Rabbit TRIM5 phylogenetically clusters with antiviral TRIM5s of other species

In addition to a rabbit TRIM5 homologue another TRIM gene located 10kb upstream of TRIM5 was identified, which showed most similarity to TRIM6 sequences from other species. Phylogenetic analyses of rabbit TRIM5 and TRIM6 revealed that the rabbit TRIM5 gene clusters together in a group with primate antiviral TRIM5 genes, the described antiviral bovine TRIM5 gene, and several related mouse genes (FIG 36). Importantly, rabbit TRIM5 clustered separately from the genes most similar to human TRIM5, i.e. TRIM34, TRIM6 and TRIM22. This strongly suggests that the rabbit TRIM5 gene, the primate TRIM5 genes and the antiviral bovine TRIM5 gene are orthologues and have a common ancestor. It also suggests that the mouse genes in this cluster may possess antiviral functions. However, a recent study showed that the *TRIM5* locus in the mouse genome comprises at least 8 different *TRIM5*–like genes. Although, none of them restricted any of the viruses analysed, which included HIV-1, SIVmac and FIV [499].

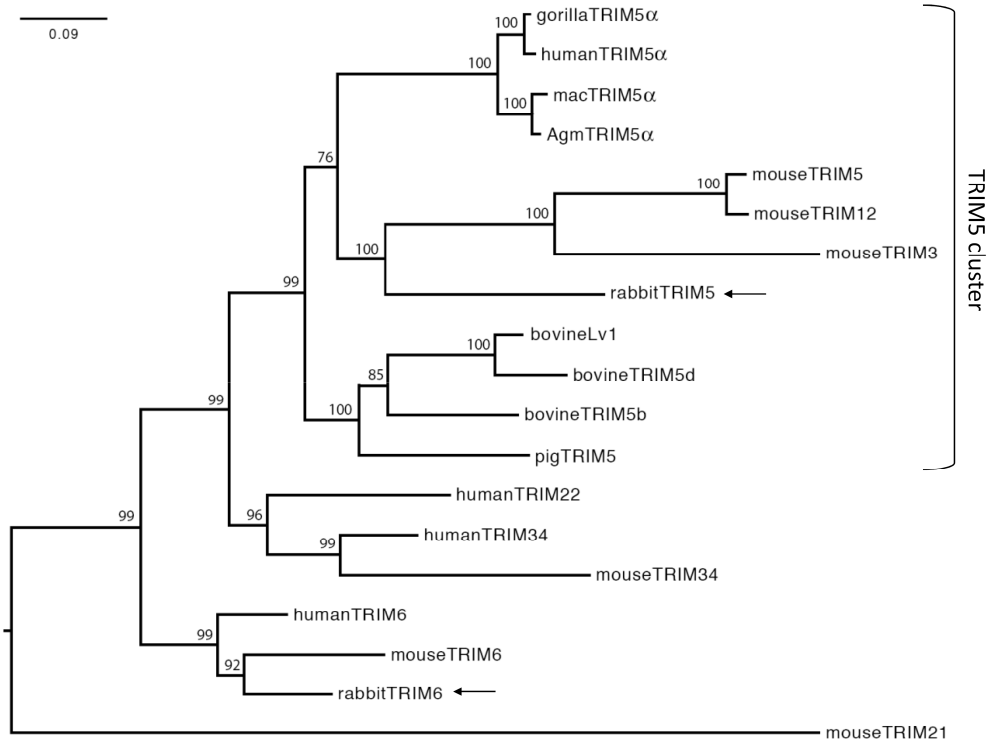


FIG 36 Phylogenetic tree of TRIM proteins from diverse species

The phylogenetic tree was created by Dr Stephane Hué by manually aligning the sequences using SeAL [478]. A maximum-likelihood phylogenetic tree was then reconstructed (see 2.2.12.). The numbers indicate the % support of 1000 bootstraps of each branch. Arrows highlight rabbit TRIM5 and TRIM6 proteins. The cluster of TRIM5 proteins is indicated.

3.2.5. Knock down of rabbit TRIM5 in SIRC cells rescues infection of restricted viruses

To test whether the block to infection in SIRC cells is due to rabbit TRIM5 expression three independent short hairpin RNA (shRNA) sequences targeting rabbit TRIM5 (shT5_1, shT5_2 and shT5_4) as well as control shRNA against the luciferase transcript (shLuc) and against rabbit TRIM6 (shT6) were designed and expressed in SIRC cells from the retroviral vector pSIREN RetroQ (see 2.2.10.). Two different approaches were followed, a transient knock down of TRIM5 (FIG 37A) and a stable knock down in a drug selected population of SIRC cells (FIG 37B). Infectious titres of HIV-1 and HIV-2 increased 3 to 10-fold in transiently transduced as well as in puromycin selected SIRC populations expressing shRNAs against TRIM5 as compared to shLuc and shT6 control cells, whereas SIVmac, MLV-B, MLV-N and EIAV titres remained unaffected. FIV has got the lowest infectious titre in SIRC cells and was rescued by 10 to 100-fold by both, transient and stable reduction of rabbit TRIM5 expression. Interestingly, the control shRNAs shLuc and shT6 rescued HIV-1 and HIV-2 infectious titres by 2-fold suggesting some non-specific effect of the MLV vector used to deliver the shRNAs.

3.2.6. Rabbit TRIM5 expressed in CRFK cells inhibits restriction sensitive viruses

To further characterise the antiviral activity of rabbit TRIM5, CRFK cells were transduced with the retroviral vector C**T**C**R** encoding rabbit **T**RIM5 and ds**R**ed. Rabbit TRIM5 expression in CTCR-transduced cells is mediated via an LTR promoter, whereas dsRed expression is under control of a CMV promoter. The transduced cells were then single cell cloned and the reddest clones (cJ and cC) selected for further studies. In both single cell clones, HIV-1 and HIV-2 infection was inhibited by 1.5 and 2.5 orders of magnitude respectively, whereas the titre of MLV-B was unaffected (FIG 38A)(data not shown). FIV infectivity was reduced by almost 3 orders of magnitude. FIV is the most sensitive virus to rabbit TRIM5, which is in agreement with the results obtained in the RNAi experiments (FIG 38A). In addition, rabbit TRIM5 was cloned into the vector EXN and expressed with an amino-terminal HA tag. Interestingly, from four analysed drug selected cell clones (c3, c6, c8 and c11) only rbT5c3 efficiently expressed HA tagged rabbit TRIM5 (FIG 38C)(data not shown). Moreover, restriction of HIV-1, HIV-2 and FIV by rbT5c3 was ~10-fold reduced compared to rbT5cC (compare FIG 38A and B). The differences in restriction of the clones tested may

retrospectively be explained by the expression of a truncated feline TRIM5 protein, which may act as a dominant negative factor against the exogenously expressed rabbit TRIM5 protein (McEwan et al, JVI, in press). In addition, expression of rabbit TRIM5 from CTCR is mediated via the LTR promoter, whereas expression from EXN is mediated via a CMV promoter. The activity of the CMV promoter is thought to be less stable over time compared to an LTR promoter, which may explain the differences in expression levels in the analysed EXN clones.

SIVmac, EIAV and MLV-N GFP vectors were also tested for their sensitivity to rabbit TRIM5 restriction (FIG 38A). Interestingly, in contrast to results obtained by shRNA mediated expression reduction in SIRC cells, EIAV and MLV-N were both restricted by exogenous expression of rabbit TRIM5 in CRFK cells by ~10 to 20-fold, respectively (compare FIG 37B with FIG 38A). This type of 'expansion' of restriction specificity by overexpression was previously observed for human TRIM5 and Fv1 [185, 286]. Importantly, infectivities of MLV-B and SIVmac were not altered indicating a rabbit TRIM5 specific effect.

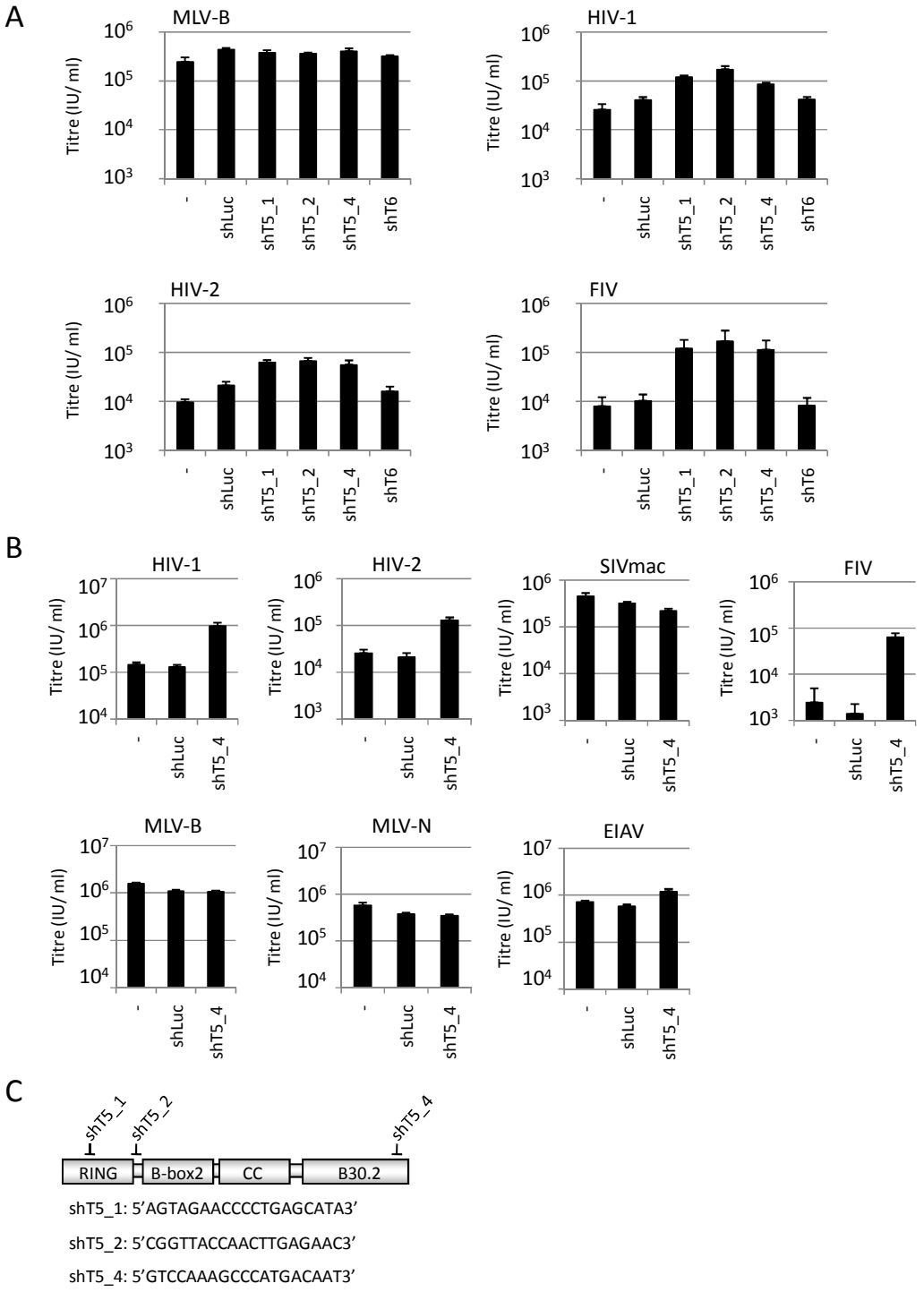


FIG 37 Transient and stable knock down of rabbit TRIM5 in SIRC cells

A) SIRC cells were transiently transduced with MLV virus like particles delivering short hairpin RNAs shT5_1, shT5_2, shT5_4 (directed against rabbit TRIM5), shLuc, or shT6 (directed against rabbit TRIM6) and challenged with the VSV-G pseudotyped GFP viruses, 48h after the first transduction. Untransduced cells were used as control. Infectious titres and standard deviations were calculated using three serially diluted viral doses. B) SIRC cells stably transduced with shLuc or shT5_4 were selected in 7µg/ml puromycin and challenged with the GFP encoding viruses shown. C) Schematic presentation of shT5 target sequences. The data are representative of two independent experiments.

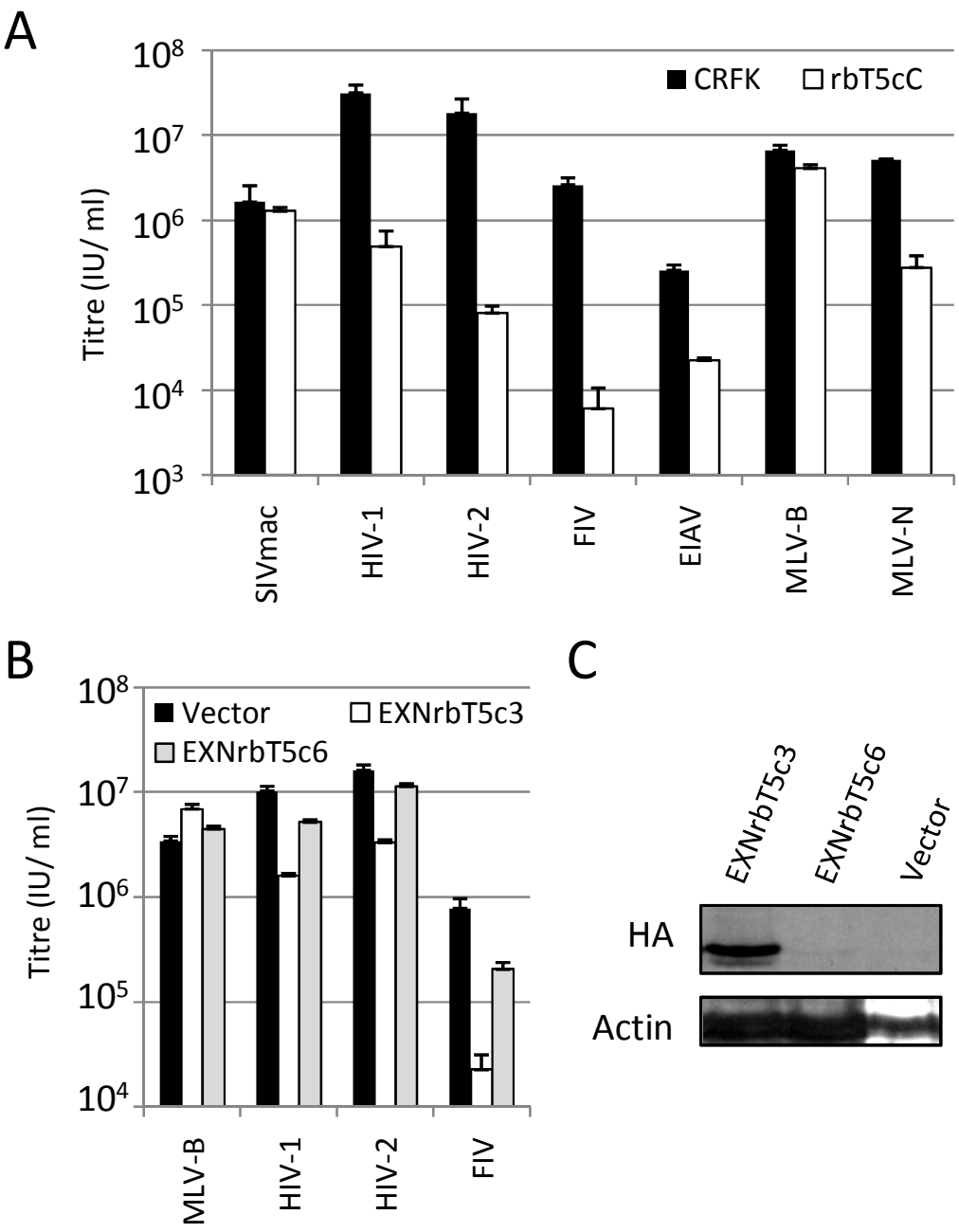


FIG 38 Exogenous expression of rabbit TRIM5 in permissive CRFK cells

CRFK cells were transduced with MLV delivering the CTCR vector encoding rabbit TRIM5 and single cell clones were challenged with GFP viral vectors indicated. Shown are the results of cell clone rbT5cC. Comparable results were obtained from the independent cell clone rbT5cJ (not shown). B) G-418 selected CRFK cell clones transduced with MLV EXN encoding rabbit TRIM5 were challenged with indicated viral vectors. C) Western blotting for HA on G-418 selected cell clones. Beta actin served as a loading control. The data are representative of two independent experiments.

3.2.7. Rabbit TRIM5 mainly blocks infection before reverse transcription

TRIM5α characteristically blocks retroviral infection very early after target cell entry and in most cases before significant reverse transcription products can be detected [184, 296, 297, 300]. However, restriction of HIV-2 by bovine TRIM5 appears not to affect the production of reverse transcripts [480]. To test at which stage rabbit TRIM5 blocks infection, rabbit TRIM5 expressing CRFK cell clones rbT5cJ and rbT5cC were infected with VSV-G pseudotyped GFP viral vectors and Taqman quantitative PCR was performed to detect the products of reverse transcription (see 2.2.9.) (FIG 39). Overexpression of rabbit TRIM5 in CRFK cells led to a significant reduction in the reverse transcription products of restricted viruses HIV-1, HIV-2, EIAV, MLV-N and FIV whereas reverse transcription of the unrestricted viruses SIVmac and MLV-B remained unaffected. In all cases the block to reverse transcription was weaker than the block to infection, an observation made in other examples of TRIM5 mediated restriction [300, 315]. This suggests that some of the restricted virus is able to reverse transcribe.

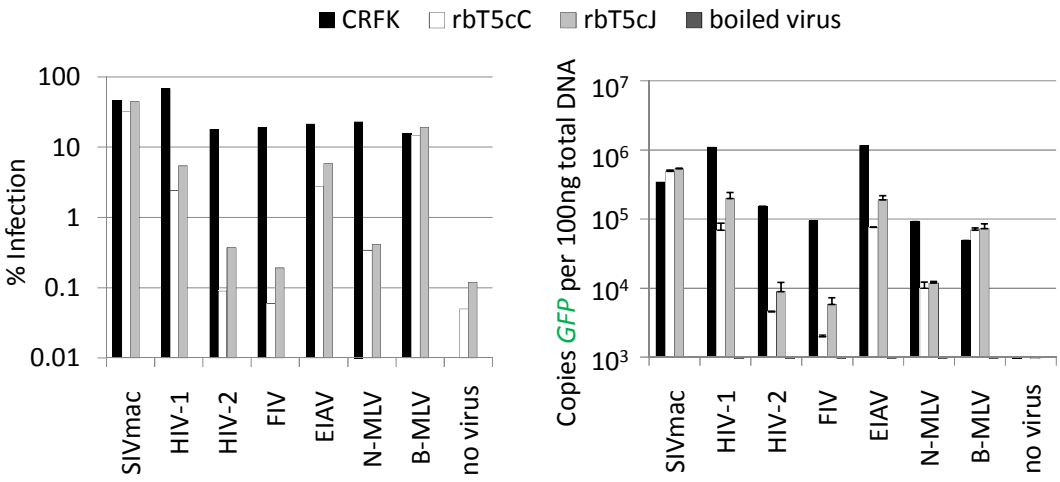


FIG 39 Reverse transcription is inhibited significantly by rabbit TRIM5

Unmodified CRFK cells or rabbit TRIM5 expressing CRFK cell clones rbT5cJ and rbT5cC were infected in triplicate with indicated GFP viral vectors and *GFP* RT products were measured by Taqman quantitative PCR on total DNA isolated 6 h after infection. One sample was used in FACS to enumerate infected cells 48 h after infection and two samples were subjected to Taqman qPCR. Depicted are *GFP* copy numbers per 100 ng total DNA. As a control for plasmid contamination cells were inoculated with boiled viral supernatants.

3.2.8. HIV-1 capsid is the target for rabbit TRIM5 restriction

It is believed that restriction depends on the binding of a dimeric TRIM5α complex to viral capsid structures via the B30.2 domain [184, 301]. Results of initial experiments comparing the permissivity of SIRC and CRFK cells to infection with SIV-HIV-1 chimaeric viruses revealed the importance of the first 146 amino acid of HIV-1 CA (FIG 33). To confirm that the capsid was the target for restriction by rabbit TRIM5 the chimaeric viruses were titrated on CRFK and rabbit TRIM5 expressing CRFK cell clone rbT5cC. HIV-1 infectious titre was reduced by almost two orders of magnitude in rbT5cC compared to parental CRFK cells, whereas HIV(S/HCA) showed no difference in titre (FIG 40). SIVmac was similarly infectious in rbT5cC cells and unmodified CRFK cells, whereas SIV(HIVCA-p2) infectious titre was reduced by two orders of magnitude in rbT5cC cells. Interestingly, both viruses HIV(H/SCA) as well as SIV(HIVCA-p2) showed infectious titres in CRFK cells that were 100-fold and 10-fold lower compared to their parental viruses, confirming previous observations and suggesting a fitness cost in fusing heterologous CA proteins [474, 498]. The data indicate that CA is the determinant for restriction by rabbit TRIM5. It suggests that the CA-dependent block observed in SIRC cells (FIG 33) is exerted by rabbit TRIM5.

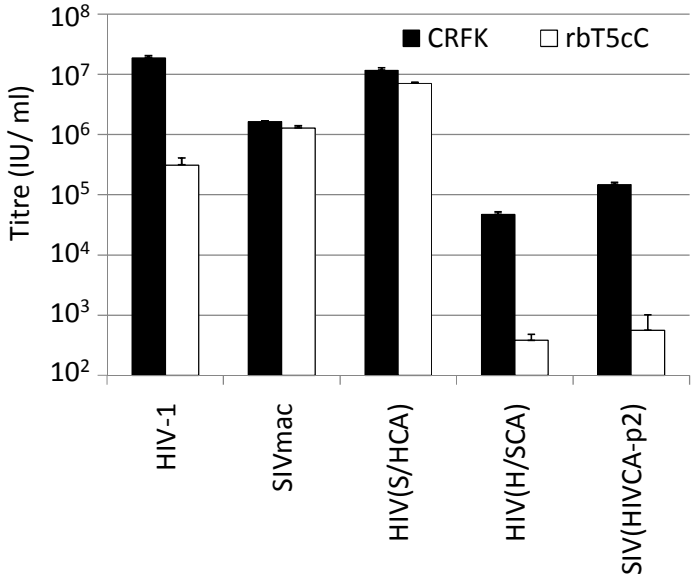


FIG 40 Titration of SIV-HIV-1 chimaeric viral GFP vectors on CRFK cells expressing rabbit TRIM5

CRFK and rbT5cC cells were challenged with VSV-G pseudotyped GFP viral vectors indicated (see also FIG 33). Infectious titres and standard deviations were calculated using three different virus doses. Error bars are standard deviations of the titre means of three different viral doses. The data are representative of two independent experiments.

3.2.9. Human TRIM5 isoforms rescue infection of restricted viruses in SIRC cells

TRIM5α restricts viruses presumably in a dimeric complex by binding to incoming capsid structures via the B30.2 domains [301]. In humans and in rhesus macaques two different splice isoforms TRIM5γ and TRIM5δ have been described to counteract the restriction activity of TRIM5α [184, 293]. In addition, human TRIM34 with a deleted B30.2 domain is able to act in a dominant negative way on the restriction by human TRIM5α [342]. Human TRIM34 also acts as a dominant negative to rhTRIM5α restriction of HIV-1 in rhesus macaque FRHK-4 cells [355]. Moreover, TRIM5 orthologues from different species can interact and alter the restriction activity of each other [500]. Since results from a recent study employing heterokaryon experiments between SIRC and human 293T cells suggested a recessive block in SIRC cells [493] it was of interest to examine whether human TRIM5 isoforms could act in a dominant negative way on retroviral restriction in SIRC cells. Therefore, SIRC cells were transduced with the CXCR retroviral expression vectors delivering human TRIM5α, TRIM5δ, or TRIM5γ (FIG 41). Transduced cells were infected with a single dose of VSV-G pseudotyped GFP encoding vectors derived from HIV-1, HIV-2, SIVmac and FIV and a two colour FACS assay was performed, as described in [293]. In this assay cells are transduced with a single dose of CXCR MLV vector so that 50% of the cells become infected. Two days later the cells are split and infected with GFP viral vectors. The percentage of CXCR transduced cells that become infected with GFP viral vectors is compared to untransduced cells by measuring both, red and green (FIG 41A). The experiment measures whether the cells expressing the short TRIM5 (which are red) are more permissive than unmodified cells (not red) in the same well (FIG 41A). Human TRIM5α or TRIM5γ rescued infectivities of HIV-1, HIV-2 and FIV in SIRC cells by two to three fold (FIG 41A). However, SIRC cells transduced with the human TRIM5δ isoform were up to 10-fold more permissive to HIV-1 and HIV-2 infection and 50-fold more permissive to infection by FIV, whereas the infectivity of unrestricted SIVmac was not altered (FIG 41A). I also calculated infectious titres on untreated SIRC cells and cells transduced with empty vector, human TRIM5α or TRIM5δ. These are expressed as fold change compared to untransduced SIRC cells (FIG 41B). The results clearly demonstrate that the human TRIM5 isoform δ can rescue infection of restricted viruses in SIRC cells. Interestingly, only human TRIM5 isoform δ but not TRIM5γ rescued infectivities of restricted viruses efficiently, suggesting that human

TRIM5δ, but not TRIM5α or TRIM5γ is able to oligomerise with the endogenous rabbit TRIM5 protein. These observations are likely to explain Cutiño-Moguel's results that human rabbit heterokaryons are permissive to HIV-1 infection [493].

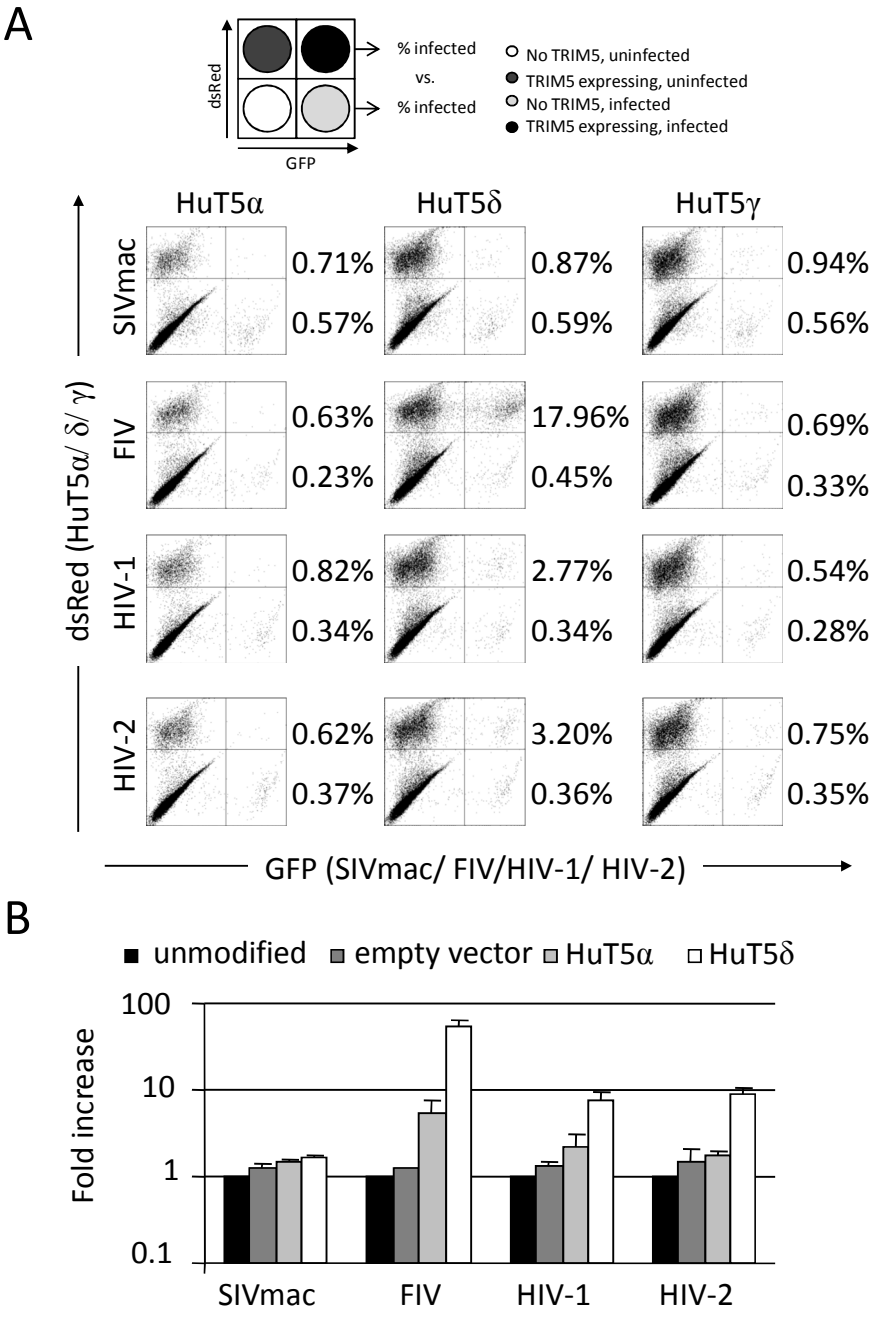


FIG 41 Dominant negative effect of human TRIM5 isoforms in SIRC cells

A) SIRC cells were transduced with MLV CTCR vector encoding human TRIM5 isoforms alpha (HuT5α), delta (HuT5δ) or gamma (HuT5γ). Transduced cells were infected with a single dose of the GFP viral vectors indicated. Infected cells were measured by FACS forty-eight hours post infection in a 2-colour assay. Percentages of GFP positive cells were calculated for CTCR transduced and untransduced cells and are indicated. B) A similar experiment as in A) was performed using three different serially diluted GFP virus doses. Fold increases in infectious titres are shown.

3.2.10. Mutation of SIVmac QQ capsid motif to HIV-2 LPA confers sensitivity to rabbit TRIM5

HIV-2 and SIVmac are related viruses, both evolved by zoonotic transmissions of SIVsm to humans and rhesus macaques, respectively. However, they have different restriction sensitivities. SIVmac is restricted by TRIM5α from squirrel monkeys whereas HIV-2 is not [481]. Alternately, HIV-2 is restricted by rhesus macaque TRIM5α but SIVmac is insensitive [481].

Moreover, HIV-2 is strongly restricted by rabbit TRIM5 whereas SIVmac is not (FIG 38). GFP viral vectors of HIV-2, SIVmac and two SIVmac CA mutants (SIV-IG/LPA and SIV-LPA) (FIG 42A) were tested for their sensitivity to rabbit TRIM5 restriction. SIV-LPA differs from SIVmac in that the wild type CA residues Q89 and Q90 were replaced by residues L89, P90 and A91, found in the HIV-2 CA at this position (FIG 42A). SIV-IG/LPA has in addition the amino acid Q85 and A87 mutated to residues found in HIV-2 (I85 and G87). Ylinen et al. showed that replacing the entire cyclophilin-binding loop in SIVmac CA with the loop of HIV-2 rendered the mutant virus sensitive to restriction by rhesus macaque TRIM5α [481]. SIV-LPA was still insensitive for restriction by rhesus TRIM5α and had lost sensitivity to restriction by squirrel monkey TRIM5α [481]. To investigate the viral determinants for HIV-2 restriction by rabbit TRIM5, HIV-2, SIVmac, SIV-LPA and SIV-IG/LPA were used to infect CRFK cells and rbT5cC cells and infectious titres were determined. I also tested the HIV-2 CypA binding loop mutant HIV-2G87A. HIV-2 as well as HIV-2G87A was strongly restricted in rbT5cC cells whereas SIVmac infectious titre was not changed compared to unmodified CRFK cells (FIG 42B). Interestingly, in contrast to wild type SIVmac, SIV-LPA was sensitive to restriction by rabbit TRIM5 and was blocked by ~50-fold (FIG 42B). Compared to the inhibition of HIV-2 infection, SIV-LPA was still ~10-fold less restricted. SIV-IG/LPA was similarly strongly restricted by rabbit TRIM5 as SIV-LPA suggesting that the residues LPA are a critical determinant that can confer sensitivity of SIVmac to rabbit TRIM5 restriction (FIG 42B). SIV-LPA was not restricted by rhesus macaque TRIM5α, suggesting that the molecular determinants for HIV-2 restriction by rabbit TRIM5 and rhesus macaque TRIM5α are slightly different.

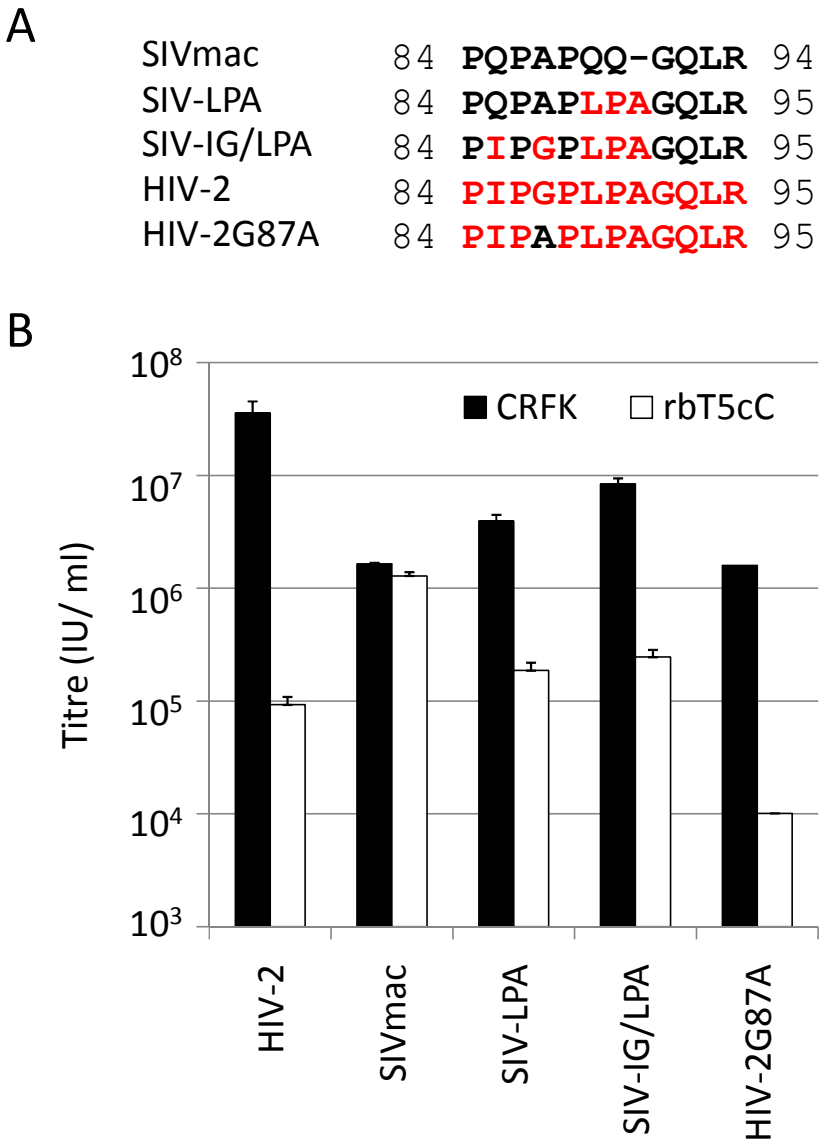


FIG 42 Analysis of restriction of HIV-2, SIVmac and SIVmac CA mutants by rabbit TRIM5

A) CA amino acid sequences of HIV-2, HIV-2G87A, SIVmac and SIVmac mutant viruses used. Numbers refer to the amino acid position in CA. B) VSV-G pseudotyped GFP encoding viral vectors were titrated on CRFK and rbT5cC cells and infectious titres were determined. Error bars are standard deviations of the titre means of three different viral doses. The data are representative of two independent experiments.

3.2.11. Hares also express a TRIM5 which restricts retroviral infections

The common ancestor of the European brown hare (*Lepus europaeus*) and the European rabbit (*Oryctolagus cuniculus*) existed approximately 12 million years ago (FIG 32). Rabbits have been shown to possess an ancient endogenous lentivirus. Moreover, finding a RELIK homologue at identical genome locations in hares suggested that this virus was circulating in a common ancestor of hares and rabbits at least 12 million years

ago [487]. The TRIM5 protein found in rabbits may have functioned as antiviral protein against this circulating lentivirus. The similarity between hares and rabbits raised the question whether hares also possess an antiviral TRIM5 protein and whether this would have similar antiviral specificities compared to rabbit TRIM5. To investigate this hypothesis, genomic DNA from a hare kidney fibroblast cell line (HareKF) (kind gift from Jean Francois Vautherot) was isolated. Primers TS82 and TS83 used to PCR amplify rabbit TRIM5 exon 8 were also used to examine the hare genome for a TRIM5 gene. The sequence of the PCR fragment amplified from hare genomic DNA showed high similarity with the B30.2 sequence of rabbit TRIM5 (FIG 43). Similarity was very high in the B30.2 β-sheets, whereas the variable regions particularly region 1 (V1) differed significantly between rabbit and hare (FIG 43) suggesting different antiviral specificities. The identification of a TRIM5-like B30.2 domain encoding sequence in the hare genome, differing significantly from rabbit TRIM5 in its variable regions, suggested selective pressure since the rabbit and hare split. To test this we compared infectious titres of viruses on hare cells with titres on CRFK cells. SIVmac and MLV-B infectious titres were similar on hare cells compared with CRFK (FIG 44A). In contrast, FIV and MLV-N showed ~40-fold reduced infectious titres in hare cells, and HIV-1 and HIV-2 were reduced by 100-fold and 400-fold, respectively (FIG 44A). The lower titre of MLV-N as compared to MLV-B strongly suggested the presence of an active antiviral TRIM5 homologue in hares. To further investigate this hypothesis the hare TRIM5 was knocked down with one of the short hairpin RNA (shT5_4) designed to knock down rabbit TRIM5 (FIG 37) which was also complementary to the hare TRIM5 exon 8 sequence. MLV vector encoding shT5_4 was used to transduce HareKF cells, which were subsequently challenged with GFP encoding vectors as before. Interestingly, shT5_4 transduced cells (HareKF T5kd) were 2 orders of magnitude more permissive for infection with MLV-N than untransduced HareKF control cells (FIG 44B). Importantly, MLV-B infectious titre was not changed. SIVmac titre was increased only moderately (5-fold), whereas HIV-1, HIV-2 and FIV titres were elevated by 20-, 50- and 90-fold, respectively. This strongly suggests that hares, like rabbits, express an antiviral TRIM5 protein. Moreover, it suggests that despite significant differences in the B30.2 variable regions, both TRIM5 proteins may have similar antiviral specificities. Further examination of the hare genome identified the entire TRIM5 gene, which is currently under further characterisation in our lab.

SIRC VHVTLTPSNNQNIVVSENKRQVMYVHYHQRLNLFSLSDDHGFRY-GTRQNYFDGILGCPS

```
Hare VHVTLTPSNNQNIVVSENKRQVMYVHYQKHGSLFLFKDNYGCQYEGIKQNYFDGILGCPS
     ****************************::: .** :.*::* :* * :************

SIRC ITSGKHYWEVDVSGKSAWILGVYGPPLLQTTTSFAYEQVSKYRPINGYWVIGLQIQYISF
Hare ITSGKHYWEVDVSGKSAWILGVYGPPSLQTIKSFAYGQFSKYRPINGYWVIGWQNQYISY
     ************************* *** .**** *.************* * ****:

SIRC EENAISLTPIVPPSRIGVFIDYEAGIVSFFSVTQHKFLIYKFSACSFSKEVFPYFNPMHC
Hare EENSICLTPIVPPSCIGVFIDYEAGIVSFFSVTQHTFLIYKFSACSFAEEVFPYFNPMQC
     ***:*.******** ********************.***********::*********:*

SIRC PKPMTICELSC
Hare PKPMTICQMSC
     *******::**
```

FIG 43 Amino acid alignment of rabbit and hare TRIM5 B30.2 domains

Genomic DNA from a hare kidney fibroblast cell line (HareKF) and SIRC cells were used to PCR amplify TRIM5 exon 8 encoding the virus binding B30.2 domain. Significant sequence differences were observed in variable regions (underlined), whereas the rest of the sequence was highly conserved.

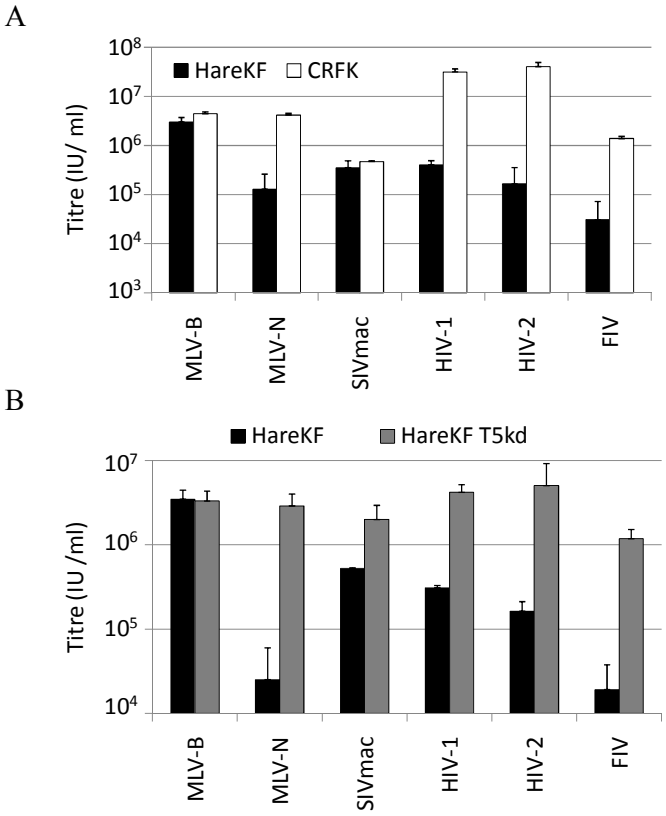


FIG 44 Hare cells restrict retroviruses likely due to expression of an active TRIM5 protein

A) CRFK cells and hare kidney fibroblasts (HareKF) were infected in parallel with VSV-G pseudotyped GFP vectors indicated and infectious titres were determined. B) HareKF cells were transduced with an MLV vector encoding a TRIM5 specific shRNA (shT5_4)(FIG 37). Stably transduced and puromycin selected cells (HareKF T5kd) and unmodified cells were infected in parallel with GFP encoding viral vectors indicated and infectious titres were determined. Error bars are standard deviations of the titre means of three different viral vector doses. The data are representative of two independent experiments.

3.2.12. The B30.2 domain of hare and rabbit TRIM5 are polymorphic

TRIM5α exhibits significant interspecies sequence variability between primates reflecting the result of adaptive evolution after positive selection from different viruses [303, 353]. Positive selection analysis of TRIM5α identified the B30.2 region v1 that is critical for species-specific restriction [303]. The interspecies sequence variability of the B30.2 variable regions is mirrored by polymorphisms in the B30.2 variable regions within a species and these polymorphisms can influence restriction properties [352, 354, 355]. Interestingly, rhesus macaques show a much higher frequency of polymorphisms in the B30.2 virus binding domain than humans (FIG 45) [354]. In general, monkeys are more polymorphic than humans. Species that constantly counteract zoonotic viruses may be under strong evolutionary pressure for changing their genome including their repertoire of restriction factors.

To study whether rabbit and hare TRIM5 are polymorphic in their B30.2 virus binding domains, genomic DNA was isolated from 6 rabbits and 3 hares and sequenced. The results are summarised in (FIG 45). Two rabbit cell lines, SIRC and EREP, encoded identical B30.2 alleles, however EREPs were heterozygous with a non-coding single nucleotide polymorphism (snp) at B30.2 nucleotide 207G/A. In addition, three out of six rabbit samples were also heterozygous at this position. In total four French farmed and two British wild rabbits were analysed, so the samples were not closely related. Two coding snps were identified in the rabbit TRIM5 B30.2 domain (V/I15 and H/Q28) (FIG 45). The V/I15 polymorphism was found in both the farmed and the wild rabbits, whereas H/Q28 was only detected in one farmed rabbit sample.

Rabbits diverged from hares ~12 million years ago (FIG 32). It is therefore interesting to compare polymorphism found in rabbit TRIM5 B30.2 domains with polymorphisms found in hares. Genomic DNA samples isolated from three different European brown hares (Hare1K-3K) as well as a hare kidney fibroblast cell line (HareKF) were used to PCR amplify hare TRIM5 exon 8. Eight snps were identified of which six were coding (V/F36, C/R58, T/I91, R/Q103, Y/F120 and C/S126), and only one of these residues (C/R58) was not located in a variable region (FIG 45). The results suggest that hares are more polymorphic than rabbits in their TRIM5 B30.2 domain, in particular in the variable regions.

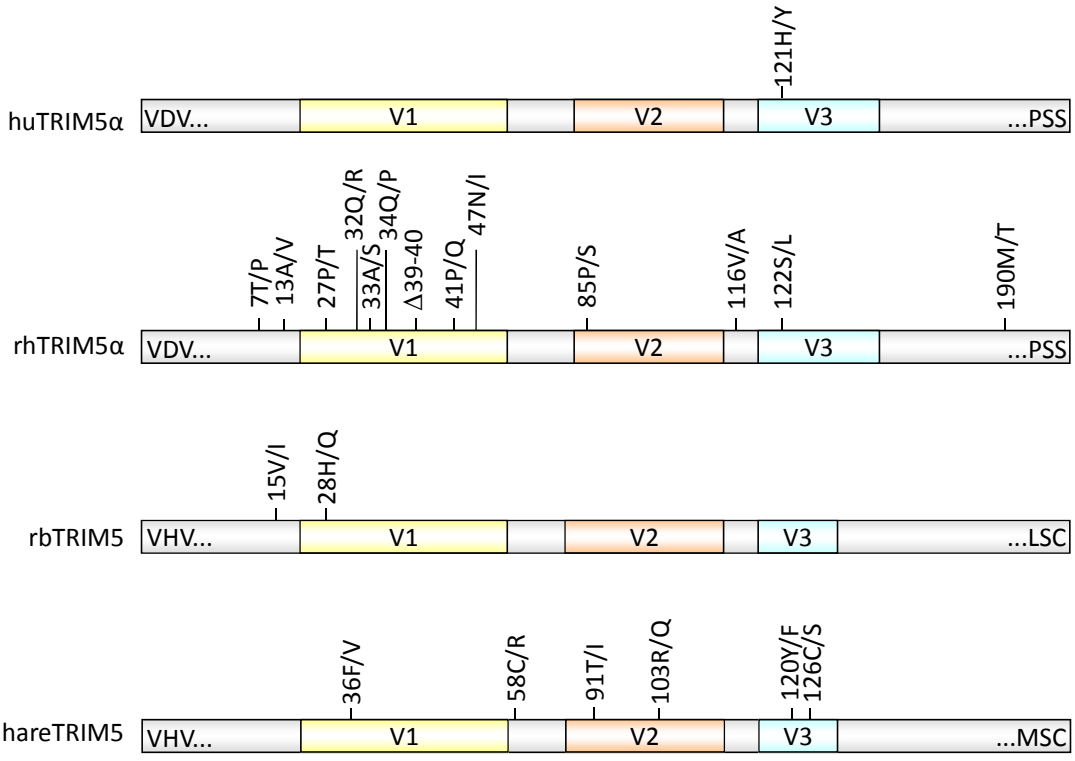


FIG 45 Summary of B30.2 polymorphisms in human, rhesus macaque, rabbit and hare TRIM5

Human TRIM5α polymorphisms were derived from [354]. Rhesus macaque TRIM5α polymorphisms were derived from [352] and [355]. Numbers reflect B30.2 amino acid residues. The first and last three amino acids of the B30.2 domains are shown. Two coding polymorphisms were identified in the rabbit TRIM5 B30.2 domain and six coding polymorphisms in the hare TRIM5 B30.2.

3.2.13. Cyclosporine rescues infection of restricted viruses in rabbit cells

In old world monkeys HIV-1 restriction by TRIM5α is strongest in the presence of active cyclophilin A (CypA) [433, 449]. Inhibiting CypA expression or function using siRNAs or the drug cyclosporine A (CsA) respectively diminishes restriction by TRIM5α, suggesting that maximal TRIM5α activity depends on CypA activity. To test whether restriction by rabbit TRIM5 is also sensitive to CypA inhibition, SIRC cells were infected with a single dose of VSV-G pseudotyped GFP encoding HIV-1, HIV-2, FIV, or SIVmac viral vectors with a CsA titration. In addition, the CsA insensitive HIV-1 CA mutant G89V and the HIV-1 SIVmac chimaeric virus HIV(S/HCA) were tested. HIV-1G89V has been demonstrated to be unable to bind CypA [426]. Infectivity of HIV-1 in SIRC cells increased after CsA treatment in a dose dependent manner by up to 16-fold for the highest dose used (10 μM), whereas the CsA insensitive mutant HIV-1G89V was only weakly increased (~3-fold) (FIG 46). Infectious titres of FIV and HIV-2 were also rescued in a dose dependent manner by up to ~15-fold and ~10-fold (for 10 μM CsA), respectively. Interestingly, SIVmac as well as HIV(S/HCA) infectious titres only increased slightly by ~3-fold at the highest dose of CsA. The data suggest that maximal restriction by rabbit TRIM5 depends on the activity of rabbit cyclophilins.

To test whether maximal restriction by endogenous rabbit TRIM5 in SIRC cells depends on rabbit Cyp activity, unmodified SIRC cells, SIRC cell bulks expressing an shRNA specific for rabbit TRIM5 (shT5_4) or the luciferase control (shLuc) were infected with SIVmac or FIV in the presence or absence of 10 μM CsA. Knock down of rabbit TRIM5 in SIRC cells rescued infectivity of FIV by ~30-fold compared to untreated or control shRNA expressing SIRC cells (FIG 47). In contrast, SIVmac infectivity was not increased. Addition of 10 μM CsA rescued infectivity of FIV in untransduced or shRLuc expressing SIRC cells by ~10-fold. A similar increase was seen in rabbit TRIM5 knock down cells, suggesting that the increase of infectivity in SIRC cells treated with CsA was independent of rabbit TRIM5 (FIG 47). However, it is likely that the rabbit TRIM5 knock down in the SIRC cell bulk was incomplete, leaving residual TRIM5 levels that were sufficient to block FIV infection. SIVmac was slightly increased in infectivity by CsA treatment in all cells by ~3-fold.

To understand, whether the increased infectivity of HIV-1, HIV-2 and FIV in SIRC cells resulted from a desensitisation to rabbit TRIM5 restriction, the same experiment was performed in CRFK cells and rabbit TRIM5 overexpressing CRFK cells (rbT5cC)

(FIG 48 and FIG 49). In rbT5cC cells HIV-1 infectivity was rescued by ~5-fold when 5 μM CsA was added and rescue was not further increased even at higher concentrations of CsA (FIG 49). In contrast, neither of the other viruses tested increased in infectivity even at the highest dose of CsA (15 μM) in rbT5cC cells. Importantly, CsA had no effect on HIV-1 or HIV-1G89V in untransduced CRFK control cells (FIG 48), strongly suggesting that the rescue of HIV-1 infectivity seen in rabbit TRIM5 overexpressing cells was specific to rabbit TRIM5 expression. Therefore, in a situation similar to that in Old World monkey TRIM5α, rabbit TRIM5 restriction of HIV-1 is maximal in the presence of Cyp activity.

Maximal infection of feline cells with FIV has been reported to be dependent on CypA since CsA treatment decreases infectivity [444]. In agreement with the published data, FIV infectivity decreased in CRFK cells by ~3 to 4 fold after CsA treatment (FIG 48). A similar effect was observed for SIVmac for the highest CsA concentration tested. The data suggest that FIV, as well as SIVmac depend on feline CypA activity for maximal infectivity, whereas HIV-1 does not. The observation that HIV-2 and FIV, which are both restricted by rabbit TRIM5, are sensitive to CsA treatment in SIRC cells but not in CRFK cells overexpressing rabbit TRIM5 remains unclear. However, since FIV as well as HIV-2 are very sensitive to rabbit TRIM5 restriction, it is likely that the high level of overexpressed rabbit TRIM5 protein overcomes a dependence on CypA activity.

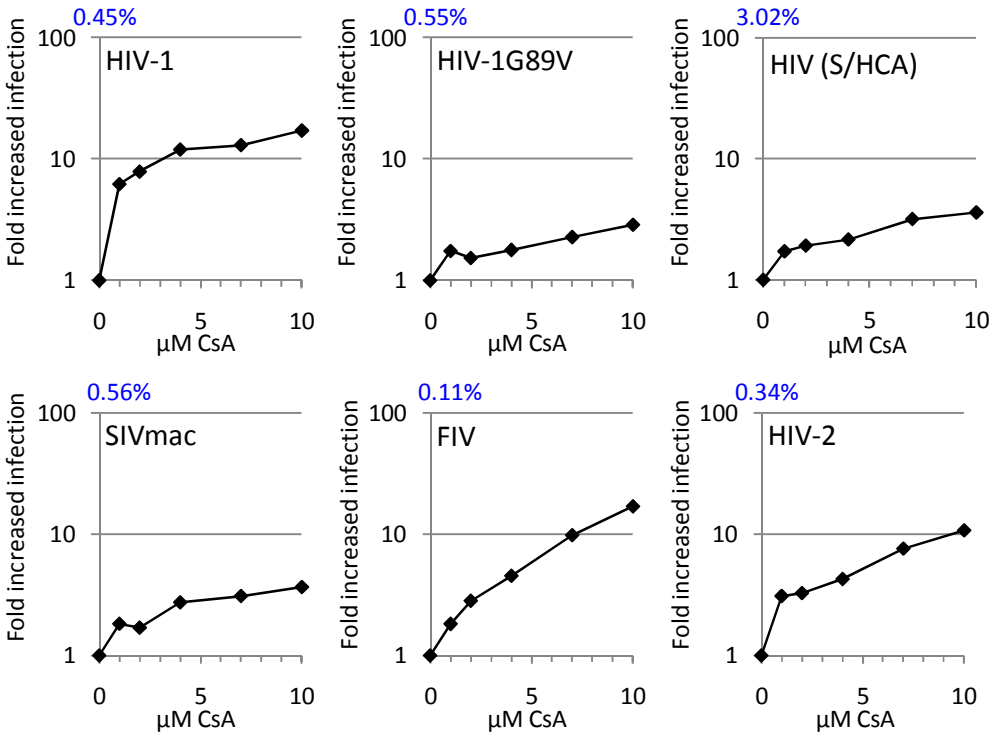


FIG 46 Titration of CsA during infection of SIRC cells with different viruses

SIRC cells were infected with a single dose of indicated VSV-G pseudotyped GFP encoding viral vectors and CsA was titrated (0-10 µM) to the infection. Graphs show fold increase of infection over the concentration of CsA added normalised to the infectivity without drug (0 µM). The percentage of infected cells without drug is given at the top of each graph. The data are representative of two independent experiments.

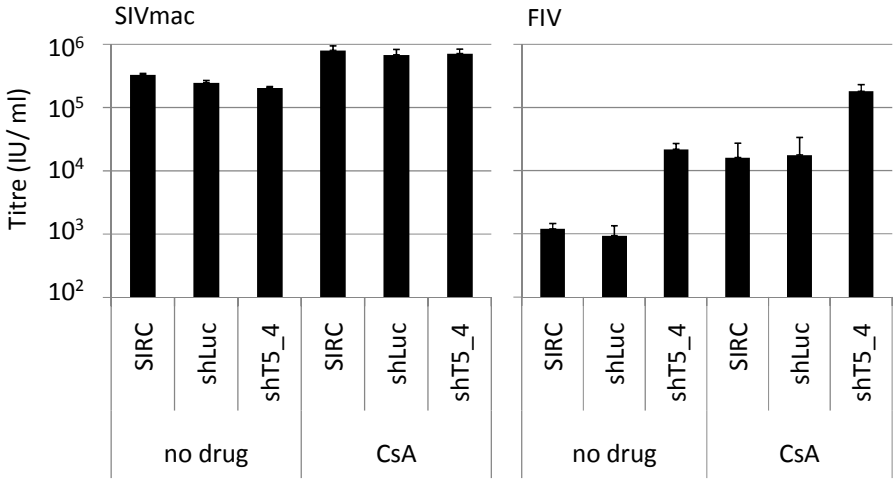


FIG 47 Effect of CsA on the restrictive phenotype of rabbit cells

Untransduced SIRC cells or SIRC cells transduced with either control (shLuc) or rabbit TRIM5 specific (shT5_4) shRNA expression vectors were infected with a serial dilution of FIV or SIVmac GFP vectors in the presence or absence of 10 µM CsA and infectious titres were calculated. Error bars are standard deviations of the titre means of three different viral doses. The data are representative of two independent experiments.

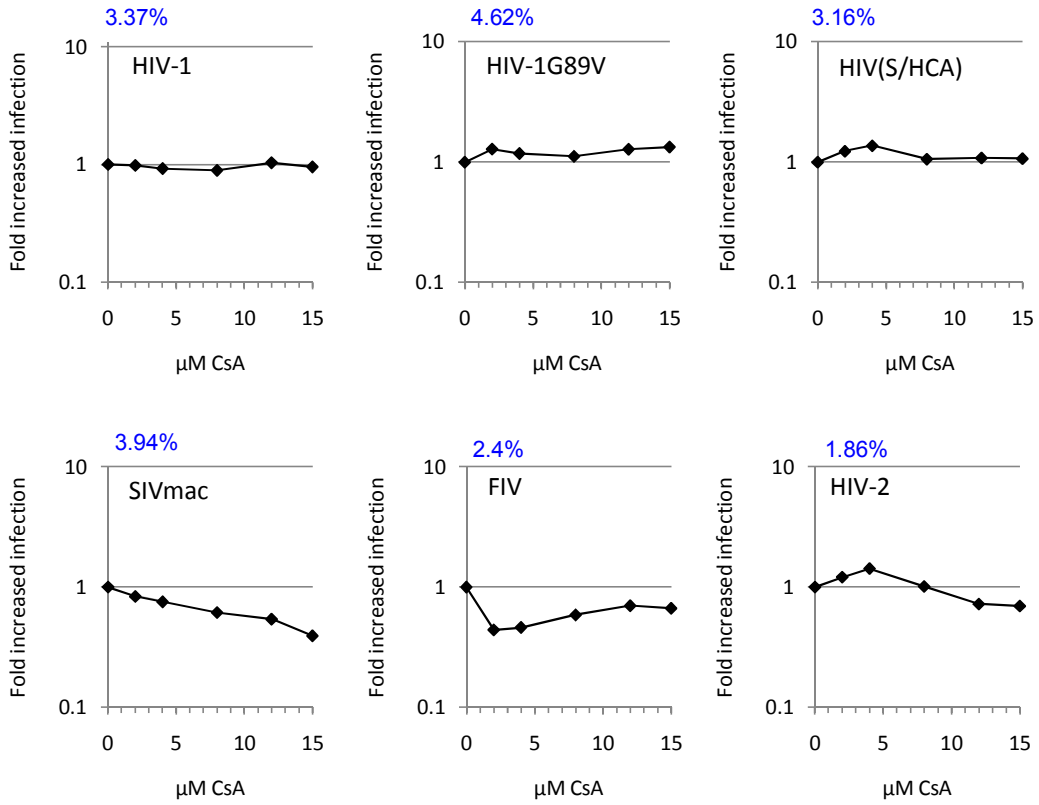


FIG 48 Titration of CsA during infection of CRFK cells with different viruses

CRFK cells were infected with a single dose of indicated VSV-G pseudotyped GFP encoding viral vectors and CsA was titrated from 0 to 15 µM to the infection. Graphs show fold increase of infection normalised to the infectivity without drug (0 µM CsA). The percentage of infected cells without drug is given at the top of each graph. The data are representative of two independent experiments.

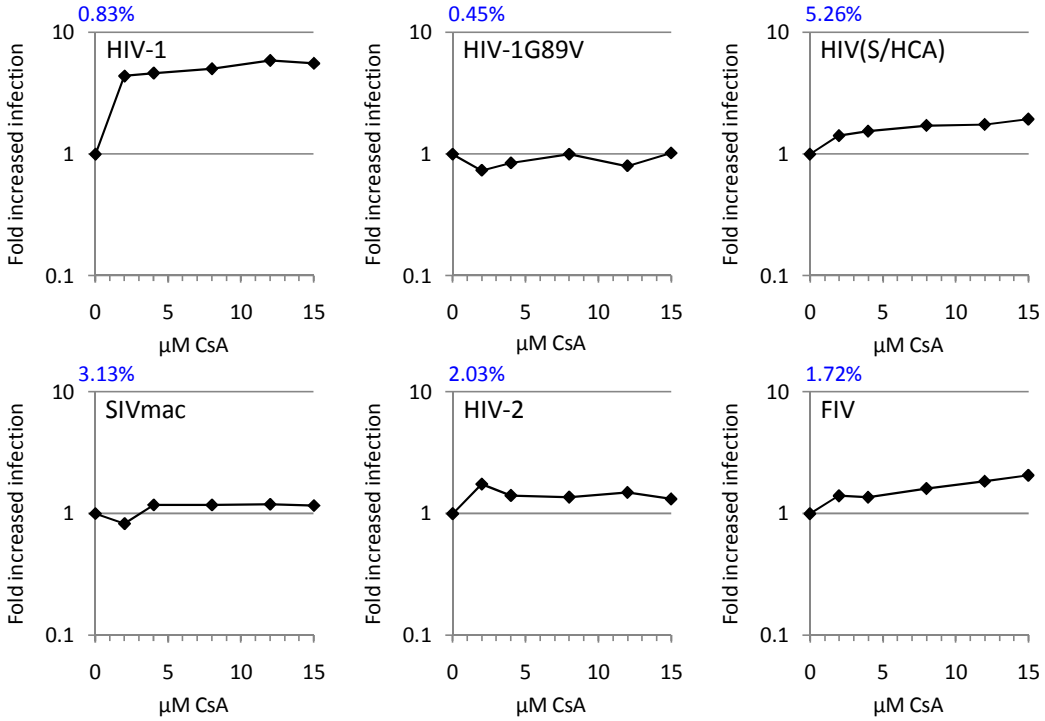


FIG 49 Titration of CsA during infection of CRFK cells expressing rabbit TRIM5

CRFK cells were infected with a single dose of indicated VSV-G pseudotyped GFP encoding viruses and CsA was titrated (0-15 µM) to the infection. Graphs show fold increase of infection normalised to the infectivity without drug (0 µM). The percentage of infected cells without drug is given at the top of each graph. The data are representative of two independent experiments.

3.2.14. Rabbit cyclophilin 18 (RbCyp18) can replace the CypA domain in TRIMCyp

The observation that CsA treatment can increase both HIV-1 and HIV-2 infectious titres in rabbit cells led to the hypothesis that rabbit CypA may be able to bind to CA proteins of both viruses. Rabbit Cyp18 (RbCyp18) is the closest rabbit protein related to CypA proteins from other species. Its amino acid sequence was aligned to the CypA sequences from owl monkey or rhesus macaque TRIMCyp, respectively and alignments are depicted in FIG 50A and B. There are several residue differences between rbCyp18 and owl monkey CypA. Notably, two amino acid residues that have been demonstrated to be important for differential restriction of HIV-1 and HIV-2 in owl monkey and rhesus TRIMCyp, D66 and R69, are conserved between owl monkey CypA and rbCyp18 (FIG 50A). So far TRIMCyp from owl monkeys has been demonstrated to potently restrict

HIV-1 but not HIV-2 [187]. Alternately, HIV-2 is restricted by rhesus TRIMCyp efficiently, whereas HIV-1 is not [186, 452]. Binding of the Cyp domain to the virus capsid is required for restriction [187], therefore measuring restriction may reveal whether a virus can bind rbCyp18 or not. To investigate whether rbCyp18 is able to bind viral CA proteins, the owl monkey TRIMCyp CypA domain was replaced with rbCyp18 (FIG 51A). A *Not*I site was engineered N-terminal to the CypA domain in owl monkey TRIMCyp and the rbCyp18 cDNA was cloned in frame to yield a fusion protein of the owl monkey TRIM5 RBCC domain to rbCyp18. CRFK cells were transduced with empty vector or vector expressing wild type owl monkey TRIMCyp, TRIMCyp with the *Not*I site (omTCypNotI), or owl monkey TRIM RBCC fused to rbCyp18 (omTRbCyp18) (FIG 51B). Drug selected cell populations were challenged with VSV-G pseudotyped GFP viruses indicated and infectious titres were determined as before in the presence or absence of CsA. Wild type owl monkey TRIMCyp as well as omTCypNotI efficiently restricted HIV-1 and FIV by ~20 to 30-fold, whereas infectious titres of SIVmac and HIV-2 were not changed. Infectivity of restricted HIV-1 and FIV could be rescued by the addition of CsA. Addition of CsA in vector control cells had a small decreasing effect on SIVmac as well as HIV-2, as shown before (FIG 48). OmTRbCyp18 potently restricted HIV-1 and FIV, as well as HIV-2 by >20-fold. Importantly, SIVmac infectivity was not increased. Addition of CsA rescued infectivity of restricted viruses almost to control levels. The data suggests that rabbit Cyp18 binds to the capsids of HIV-1, HIV-2 and FIV, when it is present in the context of a TRIMCyp protein. Rabbit cyclophilin 18 may therefore be the first naturally occurring Cyp described, that binds all three viruses, HIV-1, HIV-2 and FIV with high affinity.

A

```
RbCyp18 MVNPTVFFDIAVDGEPLGRVSFELFADKVPKTAENFRALSTGEKGFGYKGSCFHR 55
omTCyp  MVNPTVFFDIAVDGEPLGRVSFELFADKVPKTAENFRALSTGEKGFGYKGSCFHR 55
        ********************************************************

RbCyp18 IIPGFMCQGGDFTRHNGTGGKSIYGEKFEDENFLLKHTGPGILSMANAGPNTNGS 110
omTCyp  IIPGFMCQGGDFTRHNGTGGKSIYGEKFDDENFILKHTGPGILSMANAGPNTNGS 110
        ****************************:****:********************

RbCyp18 QFFICTAKTEWLDGKHVVFGRVKEGMSIVEAMEHFGSENGKTSKKITIANCGQL  164
omTCyp  QFFICTAKTEWLDGKHVVFGKVKEGMNVVEAMERFGCRYGKTSKKITIADCGQL  164
        ********************:*****.:*****:**.. **********:****
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B

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RbCyp18 MVNPTVFFDIAVDGEPLGRVSFELFADKVPKTAENFRALSTGEKGFGYKGSCFHR 55
rhTCyp  MVNPTVFFDIAVDGEPLGRVSFELFADKVPKTAENFRALSTGEKGFGYKGSCFHR 55
        ********************************************************

RbCyp18 IIPGFMCQGGDFTRHNGTGGKSIYGEKFEDENFLLKHTGPGILSMANAGPNTNGS 110
rhTCyp  IIPGFMCQGGNFTHHNGTGGKSIYGEKFEDENFILKHTGPGILSMANAGPNTNGS 110
        **********:**:********************:********************

RbCyp18 QFFICTAKTEWLDGKHVVFGRVKEGMSIVEAMEHFGSENGKTSKKITIANCGQL- 164
rhTCyp  QFFICTAKTEWLDGKHVVFGKVKEGMNIVEAMERFGSRNGKTSKKITIADCGQLE 165
        ********************:*****.******:***.***********:****
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FIG 50 Amino acid alignment of rbCyp18 with owl monkey and rhesus TRIMCyp CypA

Amino acid alignment of rabbit cyclophilin 18 (RbCyp18) and CypA of TRIMCyp from A) *Aotus trivirgatus* (omTCyp) or B) *Macacca mulatta* (rhTCyp). (*) identical, (:) similar, or (.) weakly similar amino acids are indicated, differences are shown in red. OmTCyp and rhTCyp residues known to be important in differential restriction of HIV-1 and HIV-2 are highlighted in yellow.

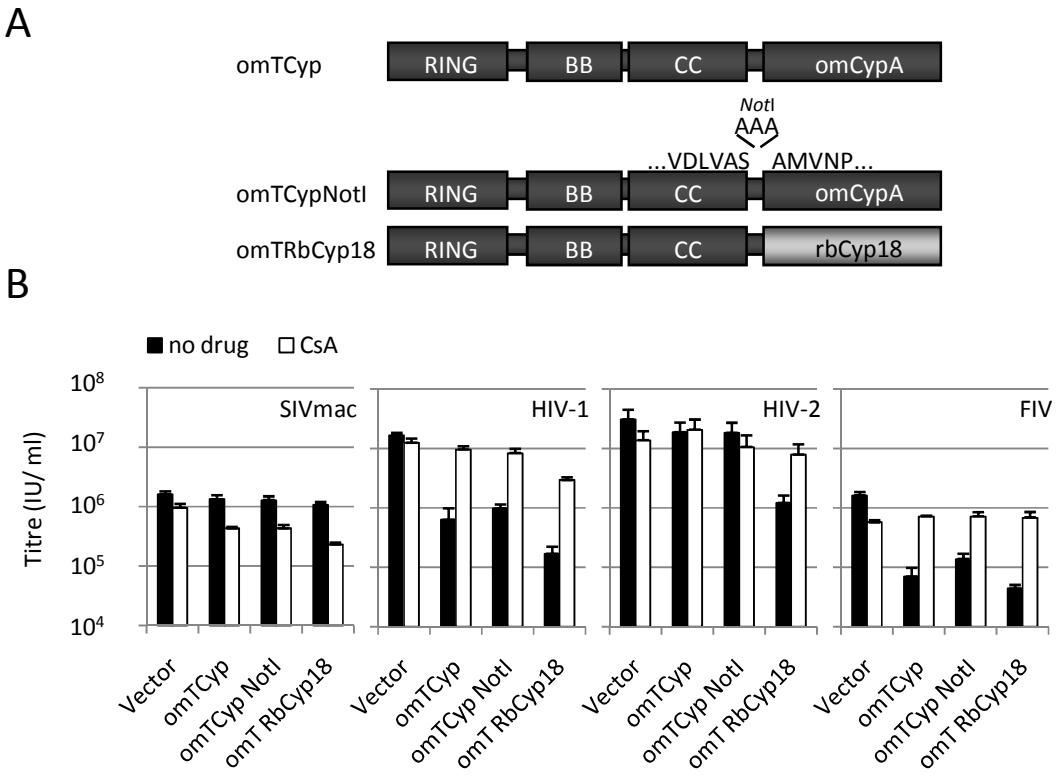

FIG 51 Rabbit Cyp18 can replace CypA in owl monkey TRIMCyp

A) A *Not*I restriction site was introduced into TRIMCyp from owl monkeys (omTCypNotI) and used to replace omCypA with Cyp18 from rabbits (rbCyp18). B) CRFK cells transduced with empty vector or vector expressing wild type owl monkey TRIMCyp (omTCyp), omTCypNotI, or omTRbCyp18 were infected with HIV-1, HIV-2, SIVmac or FIV GFP vectors in the presence or absence of 5μM cyclosporine (CsA). Infectious titres were calculated on three different doses of serial diluted virus. Error bars are standard deviations of the titre means of three different viral doses. The data are representative of two independent experiments.

3.3. Discussion

Rabbit cells are poorly permissive to infection by a variety of retroviruses including HIV-1 [297, 494]. I show here that SIRC cells are specifically less permissive for some viruses (HIV-1, HIV-2 and FIV) than for other viruses (e.g. SIVmac). This conclusion was drawn by comparison of infectious titers on SIRC cells and CRFK cells using the same virus stocks. Therefore the observed difference in the reduced permissivity on SIRC cells as compared to CRFK was likely an effect of a cell specific factor. However, it would be useful to compare the virus stocks by RT-ELISA to ensure that the difference in infectivity is due to specific restriction and not difference in virus dose. This will allow normalising the results to the amount of RT, a.e. infectious particles, added to the cells. I expect that this will support the notion that specific viruses (e.g. HIV-1, HIV-2 and FIV) are sensitive to restriction by rabbit TRIM5, whereas others (e.g. SIVmac) are not.

The low permissivity of SIRC cells can be at least partially explained by the expression of a TRIM5 protein that restricts infection by some retroviruses. Rabbit TRIM5 most potently restricts FIV, whereas HIV-1 and HIV-2 are moderately restricted depending on the experimental system. Importantly, SIVmac is not restricted by rabbit TRIM5 and acts as an important control.

The identification of rabbit TRIM5 enabled us to analyse the phylogenetic relationship between antiretroviral TRIM proteins in more detail. In addition to primate TRIM5 proteins, recent studies identified an active antiviral TRIM5α protein in cattle [480, 501]. The bovine genome harbours more than one TRIM5 gene suggesting the expression of multiple different TRIM5s but only one of them has been demonstrated to possess antiviral characteristics. It is interesting to consider whether TRIM5 genes in different species have evolved from a common ancestral TRIM5 gene, possibly possessing antiretroviral activity, or whether they have evolved independently from each other as suggested by a recent study [501]. Independent evolution of antiviral TRIM5 genes implies that they have evolved separately from each other as a response to retroviral infections. On the other hand, convergent evolution describes a common TRIM5 ancestor that possibly had antiviral properties and was evolutionary inherited from one species to the next.

With the rabbit TRIM5 in hand the phylogeny of mammalian TRIM5s could be investigated in more detail. Consideration of a phylogenetic tree showed that rabbit

TRIM5 clusters with antiretroviral TRIM proteins of cattle and primates including human TRIM5. Importantly, the closest human TRIM5 genes TRIM34, TRIM22 and TRIM6 clustered independently of all the antiviral TRIM5 genes indicating that the rabbit TRIM5 is a true orthologue of human TRIM5 and probably evolved from a common ancestral TRIM5 gene. It seems likely that this ancestral protein had antiretroviral activity. This stands in contrasts to a recently published paper which suggests that TRIM5 genes have evolved independently in each species [501]. The presented data clearly show that rabbit TRIM5 is a true orthologue and clusters with other antiviral TRIM5 proteins. Interestingly, a recent study investigated the TRIM5 gene locus in rodents and suggested that members of this cluster may exert antiviral activity, although the authors did not observe restriction against any of the viruses tested, including HIV-1 and FIV [499]. In addition, it is also interesting that some species like cattle and rodents, demonstrate a significant expansion of their TRIM5 locus, suggesting that they have been under evolutionary pressure maybe from circulating viruses [480]. In contrast some species have lost parts of their TRIM5 gene (cats) or even the whole TRIM5 gene (dogs) [374, 502]. Analysis of the available rabbit genome by BLAST identified no further genes homologous to rabbit TRIM5, suggesting that it is not expanded as it is in cattle or rodents. The closest TRIM gene identified in the rabbit genome, clustered with TRIM6 proteins of other species, therefore appears to be a TRIM6 orthologue. Moreover, PCR using TRIM5 specific primers never resulted in more than two sequences in one individual, further suggesting that the identified gene is the only TRIM5 gene in the rabbit genome.

Reduction of rabbit TRIM5 expression in SIRC cells using TRIM5 specific shRNAs partially rescued the titres of restricted viruses and overexpression of rabbit TRIM5 in otherwise permissive feline cells rendered them non-permissive, specifically to viruses that were also restricted in SIRC cells. Of all viruses tested, FIV appeared to be most sensitive to rabbit TRIM5 and was rescued best in RNAi experiments. Rabbit TRIM5 RNAi experiments were performed with drug selected bulks of SIRC cells transduced with vectors encoding independent shRNAs directed against rabbit TRIM5. Transduced cell pools consistently grew poorly under drug selection (data not shown) suggesting that knocking down TRIM5 was not well tolerated in SIRC cells. Furthermore, CRFK cell pools transduced with rabbit TRIM5 expression vectors also often showed low expression levels of rabbit TRIM5. However, CRFK cell clones overexpressing rabbit TRIM5 could be generated, which were strongly non-permissive to infection by HIV-1,

HIV-2, EIAV and FIV. Importantly, the infectivity of SIVmac was unaffected in these clones. Strikingly, overexpression of rabbit TRIM5 resulted in the restriction of MLV-N but not MLV-B whereas the reduction of rabbit TRIM5 in SIRC cells had no effect on MLV-N infectivity. A similar expansion of specificity after overexpression has also been observed for human TRIM5α and Fv1 [286, 481].

Rabbit TRIM5 shares characteristics with antiviral TRIM5 proteins from primates. It restricts before significant amounts of viral DNA are generated by reverse transcription and can be saturated by short isoforms of human TRIM5. This observation may explain the results of Cutiño-Moguel & Fassati, who demonstrated that heterokaryons between human and rabbit cells are permissive to HIV-1 infection [493]. The authors suggested that an important factor for intracellular HIV-1 RTC trafficking to the nucleus is lacking in SIRC cells [493]. The most compelling observation in this study relies on a heterokaryon experiment, in which 293T cells were fused with SIRC cells. The resulting heterokaryons were susceptible to infection by VSV-G pseudotyped HIV-1 vector, which suggested that the 293T cells provided a missing factor(s) for replication in SIRC cells. However, studies investigating the differential expression of TRIM5 isoforms in 293T cells suggested that they strongly express human TRIM5 isoform delta (Ben Webb, unpublished). The elevated levels of TRIM5δ may explain the high permissivity of these cells to a variety of diverse retroviruses, including MLV-N, by counteracting endogenous TRIM5α levels. In this study, I demonstrated that HIV-1 infectious titre is rescued in SIRC cells by the exogenous expression of human TRIM5δ. Fusing 293T cells with SIRC cells may have a similar effect in that rabbit TRIM5 is saturated by the human isoform delta, explaining the increase of HIV-1 infectivity in heterokaryon cells observed by Cutiño-Moguel & Fassati. However, the possibility of a lacking factor in addition to the antiviral TRIM5 cannot be excluded at the moment and may be supported by the observation that even in SIRC cells transduced with rabbit TRIM5 specific shRNAs infectious titres of restricted as well as unrestricted viruses always were >10-fold lower than in other cells, e.g. CRFK (FIG 33).

The target for primate and non-primate antiviral TRIM5 proteins is the CA protein [184, 185, 299, 480, 503]. Likewise, rabbit TRIM5 restricts viruses depending on their CA sequences. HIV-1 is rendered insensitive to rabbit TRIM5 when the N-terminal CA from SIVmac is used to replace the corresponding HIV-1 sequence. Moreover, SIVmac can be rendered sensitive to restriction by rabbit TRIM5 when two CA amino acids (Q89Q90) are replaced by the HIV-2 residues (LPA). Importantly, this SIVmac mutant

loses the sensitivity to restriction by squirrel monkey TRIM5α and at the same time remains insensitive to restriction by rhesus macaque TRIM5α [481]. The observations support the notion that these CA residues impact on TRIM5 sensitivity and that changes can confer sensitivity as well as insensitivity to TRIM5 proteins of different species.

The partial rescue in infectivity of restricted viruses could also be explained by another yet unidentified rabbit restriction factor or as suggested by another study due to the lack of a positive factor [493]. In this respect, it is interesting that addition of CsA during infection rescued the titre of restricted FIV in SIRC cells to a similar extent as in SIRC cells with reduced rabbit TRIM5 levels. However, a likely explanation for this observation is that rabbit TRIM5 levels were not completely reduced and that residual TRIM5 was still able to restrict FIV.

Several studies have shown, that cyclophilin A is a factor enhancing HIV-1 restriction by old world monkey TRIM5α and that treatment with cyclosporine A increases HIV-1 titres [433, 448, 449]. Rabbit cyclophilin 18, the cyclophilin with the highest amino acid similarity to CypA from other species, may act in an identical way to enhance restriction by rabbit TRIM5. The presented data support this model for HIV-1. Infectivity of HIV-1 in CRFK cells overexpressing rabbit TRIM5 can be partially rescued by the addition of CsA. Moreover, SIRC cells can be rendered up to 16-fold more permissive for HIV-1 but not HIV-1G89V which does not bind CypA, when CsA is added during infection. These observations are similar to results obtained with old world monkey TRIM5α from rhesus macaques or African green monkeys [433]. The data supports the model in which rabbit TRIM5, like Old World monkey TRIM5s, preferentially binds to one isomeric state of the peptide bond at G89-P90, e.g. *trans* (FIG 52). Rabbit Cyp18 catalyses isomerisation which leads to more *trans* peptide bonds available to bind to rabbit TRIM5 as it takes the rare form out of the reaction. Disruption of rabbit Cyp18 activity by CsA addition decelerates the reactions between the *cis* and the *trans* state leading to less available G89-P90 *trans* peptide bonds for binding to rabbit TRIM5. In the case of overexpression of rabbit TRIM5, the high levels of exogenous TRIM5 protein may cause a sink so that the reaction from *cis* to *trans* is enhanced. Therefore, CsA addition does not lead to a complete rescue of restricted virus.

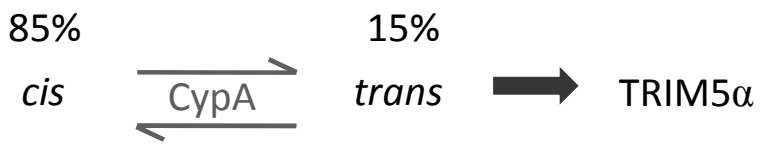

FIG 52 Mechanism of CypA activity on TRIM5 restriction

A) CypA catalyses isomerisation of the HIV-1 CA peptide bond G89-P90. TRIM5α binds preferentially one isomeric form (e.g. *trans*). In the presence of CypA an equilibrium is build and 85% of G89-P90 peptide bonds are found in *cis* and 15% in *trans*. In the absence of CypA the isomerisation is not catalysed leading to less available *trans* isomers for binding to TRIM5α, therefore more viruses can infect the cell, i.e. restriction is decreased.

Interestingly, rabbit Cyp18 appears to be able to bind to diverse lentiviral capsids including HIV-1, HIV-2 and FIV. This observation was made by fusing rabbit Cyp18 to the owl monkey RBCC domain and analysing restriction of viruses by that chimaera. This strategy assumes that dimeric binding specificity (in the context of a TRIMCyp molecule) is similar to monomeric binding specificity (in the context of free CypA proteins), which is unlikely. However, thus far no case is known in which a TRIMCyp binds to capsid but the corresponding Cyp monomer does not. Analysis of monomeric rabbit Cyp18 binding to HIV-1 and HIV-2 capsids are currently being performed by collaborators and will reveal whether monomeric rabbit Cyp18 is able to bind viral capsids. However, in context of the TRIMCyp with the rabbit Cyp18 domain, infectivities of restricted viruses could be rescued with CsA. It is therefore possible that treatment of SIRC cells with CsA also leads to inhibition of monomeric rbCyp18 which may directly or indirectly explain the increase in infectivity seen for HIV-1, HIV-2 or FIV as described by the model above.

In addition to rabbits, hares also possess an antiretroviral TRIM5 gene. The study shows that kidney fibroblasts from hares are significantly less permissive to infections by HIV-1, HIV-2, FIV and MLV-N GFP vectors than by SIVmac or MLV-B. Interestingly, MLV-N is significantly reduced in titre as compared to the closely related MLV-B, suggesting that hares have an antiretroviral TRIM5 gene able to restrict MLV-N. Amplification of a putative TRIM5 exon 8 from hare genomic DNA, enabled the investigation of this gene by shRNA mediated knock down. Expression of a short hairpin RNA specific to the exon 8 sequence rescued infectivities of restricted viruses to levels in unmodified cells. This strongly suggests the presence of an active antiretroviral hare TRIM5 protein, although the full-length cDNA should be cloned and tested. The B30.2 sequence from the putative hare TRIM5 shows strong conservation with the

rabbit TRIM5 B30.2 domain. It however differs significantly in the V1 region. The last common ancestor of rabbits and hares existed ~12 million years ago. The European rabbit and the European brown hare both posses homologues of an endogenous lentivirus type K, RELIK, integrated at identical sites in their genomes, suggesting that this virus was present before the divergence of hares and rabbits [486, 487] (FIG 32). One may speculate that the ancestor of RELIK was still an exogenous, replicating virus 12 million years ago. The differences found in the variable regions of the TRIM5 B30.2 domains in rabbits and hares therefore may reflect the struggle of one or the other species to combat this virus. Several polymorphisms were identified in the hare TRIM5 B30.2, whereas only two polymorphisms were found in rabbits. It is possible that these polymorphisms relate to different antiviral specificities since most of them were located in the variable regions.

Rabbits have been proposed as a surrogate animal model for HIV/ AIDS and some studies suggested that HIV-1 will replicate in rabbits when either HIV-1 virus or chronically infected human H9 cells are injected into rabbits [488, 490, 491]. However, despite promising early results a persistent infection in those animals could not be achieved. In fact, isolated rabbit cells have been shown to be generally poorly permissive to HIV-1 infection [297, 493, 494]. The poor permissivity results from several barriers including the lack of a functional receptor/ co-receptor complex. One study suggests that this entry barrier can be bypassed by exogenous expression of CD4 and CCR5 in SIRC cells [504]. In this respect, the results in the present study suggest that knock out of rabbit TRIM5 might be an important feature of the further development of rabbits as an animal model for HIV/AIDS.

4. Characterisation of Cyp fusion proteins as restriction factors

4.1. Introduction

To date the owl monkey and macaque TRIMCyps are the only natural TRIM5 CypA fusion proteins found. However, it is unclear what advantage TRIM5 provides over other TRIM proteins and why CypA is preferred over other cyclophilins. To address the question why cyclophilin A has fused to TRIM5 twice in evolution, the potential of other cyclophilins, namely human CypA, CypB, CypC, CypE, CypF and the Cyp domain of Nup358 (Nup358Cyp), to replace the CypA domain in owl monkey TRIMCyp was investigated. Understanding this may reveal important insights into the advantage cyclophilin A provides over other cyclophilins. It may as well identify other cyclophilins that can target the HIV-1 CA and exert a block to infection when fused to the TRIM5 RBCC motif. To address the question of why TRIM5 was twice selected, the ability of other TRIM family members to provide the RBCC motif to form an active TRIMCyp, was investigated. Furthermore, the unrelated mouse restriction factor Fv1 was fused to CypA to examine whether the enzymatic function of the RBCC motif in TRIMCyp is required for restriction.

Human CypA and CypB have been demonstrated to bind HIV-1 Gag and CA protein [422]. Nup358 has recently been suggested to be involved in nuclear entry of HIV-1 pre-integration complexes [107]. König et al. showed that RNAi mediated reduction of levels of Nup358 or TNPO3, which is thought to bind to Nup358 and is involved in nuclear entry, decreases HIV-1 infection, suggesting that HIV-1 uses both proteins to access the nucleus. Nup358 is a 3324 amino acid long protein consisting of several domains including a C-terminal Cyp like domain. It is found in nuclear pore complexes and is believed to be involved in transportin-dependent nuclear entry [505]. A recent report proposes that the HIV-1 Rev protein is imported into the nucleus in a Nup358 and transportin dependent way, suggesting that Nup358 may be a key player in both HIV-1 PIC nuclear import, as well as nuclear import of important viral regulators, like the Rev protein [506]. Nup358 interacts with several proteins including kinesin-1, KIF5B/KIF5C, HDAC4, Ubc9, RanGAP1 and others and is thought to mediate nuclear import of substrate receptor complexes [507]. Some studies also suggest that it might recruit proteasomal subunits to nuclear pore complexes [508]. It was proposed that Nup358 builds the filaments that are localised to the cytoplasmic site of the NPC,

suggesting that it ‘catches’ substrates that need to be modified and/ or imported [509]. Interestingly, a study in Drosophila melanogaster suggested that the cyclophilin domain and the Ran-binding domain 4 (RBD4) of Nup358 act together as a supradomain as a chaperone on red/ green opsin [510]. It is not hard to envision that HIV-1 binding to the Nup358Cyp domain might also induce a chaperone function of Nup358 supporting either uncoating and/ or nuclear import. In this respect it is noteworthy that HIV-1 uncoating has recently been suggested to occur at sites close to the nuclear membrane [100].

Nup358 may also be involved in Crm1 mediated nuclear export [511]. Furthermore, Nup358 is target for sumoylation, which has been demonstrated to be important for efficient association with RanGTPase activation protein 1 (RanGAP1) [512, 513]. In addition, Nup358 itself is an E3 SUMO ligase that mediates sumoylation of substrates, e.g. HDAC4 that is involved in transcriptional repression [514, 515]. In addition to nuclear import, recent data also reveals an important role for Nup358 in the resolution of sister chromatides during cell division [516].

4.2. Results

4.2.1. Human CypA, B and E and the Cyp domain of Nup358 bind lentiviral capsids

To test the hypothesis that other cyclophilins may also be able to recruit the RBCC motif to viral capsids when fused to TRIM5, owl monkey TRIMCyp (omTCyp) with a *Not*I site between the RBCC motif and the CypA moiety was engineered. This modified TRIMCyp protein restricted HIV-1 to similar levels as wild type owl monkey TRIMCyp (FIG 55A). Human CypA, CypB, CypC, CypE, CypF and the cyclophilin domain from the nuclear pore protein Nup358 (FIG 53) were used to replace the owl monkey CypA moiety, creating the fusion proteins omThuCypA/B/C/E/F and omTNup358Cyp. An amino acid alignment of the catalytic domains of the Cyps tested indicates that residues that have been shown to be important for human CypA substrate binding are relatively conserved between the different Cyps.

All owl monkey TRIM human Cyp fusion proteins were expressed in CRFK cells and drug selected cell populations were investigated for their permissivity to infection by HIV-1, HIV-2, SIVmac, or FIV (FIG 55). Wild type owl monkey TRIMCyp served as a positive control, whereas owl monkey TRIMCyp with a deleted CypA domain was used as a negative control. Wild type omTCyp, omTCypNotI as well as omThuCypA potently restricted HIV-1 and FIV infectivity by ~50-fold, whereas SIVmac was unrestricted (FIG 55). In fact, SIVmac showed no sensitivity to any of the tested fusion proteins. Interestingly, HIV-2 was very weakly (2-fold) restricted by wild type omTCyp, whereas omThuCypA restricted HIV-2 more potently by ~8-fold (FIG 55). OmThuCypB only restricted HIV-1 and the block was very weak ~2 to 3-fold. OmTCypE blocked infectivity of HIV-1 and FIV similarly by 6 to 8-fold, whereas HIV-2 was not affected. Interestingly, despite moderate amino acid conservation between Nup358Cyp and huCypA (FIG 54), omTNup358Cyp restricted HIV-1, HIV-2 as well as FIV by 6 to 8-fold. OmThuCypC and omThuCypF showed no restriction against the tested viruses. To analyse the expression levels of the proteins, the N-terminal HA tag was detected by western blotting of cell extracts (FIG 56). As a loading control beta actin levels were measured on stripped blots. The western blot shows similar expression levels of the expressed proteins, although omTCypNotI expression was slightly lower than the other proteins.

Next, drug selected cell clones of the transduced CRFK cell populations were infected with HIV-1 in the presence or absence of 5 µM CsA and infectious titres were determined. The single cell clones recapitulated results obtained from the bulk populations in terms of restriction capacity (FIG 55 and FIG 57). Interestingly, HIV-1 infectivity was rescued in all cases apart from omTNup358Cyp by the addition of 5 µM CsA, suggesting that Nup358Cyp does, if at all, only weakly bind CsA. Several amino acids shown to be important for human CypA interaction with cyclosporine A are different in Nup358Cyp suggesting that the sensitivity to CsA treatment may not be the same as that of CypA [421] (FIG 26 and FIG 54).

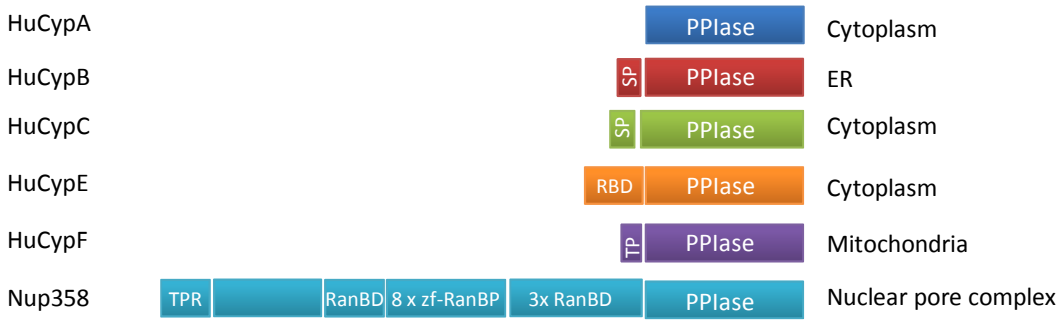

FIG 53 Schematic representation of different Cyps analysed in the context of TRIMCyp
PPIase, peptidyl-prolyl-isomerase; SP, signal peptide sequence; RBD, RNA binding domain; TP, transition peptide; TPR, Tetratricopeptide repeat domain; RanBD, Ran binding domain; zf-RanBP, zinc finger in Ran binding protein; ER, endoplasmic reticulum; Subcellular compartments to which the Cyps localise are indicated.

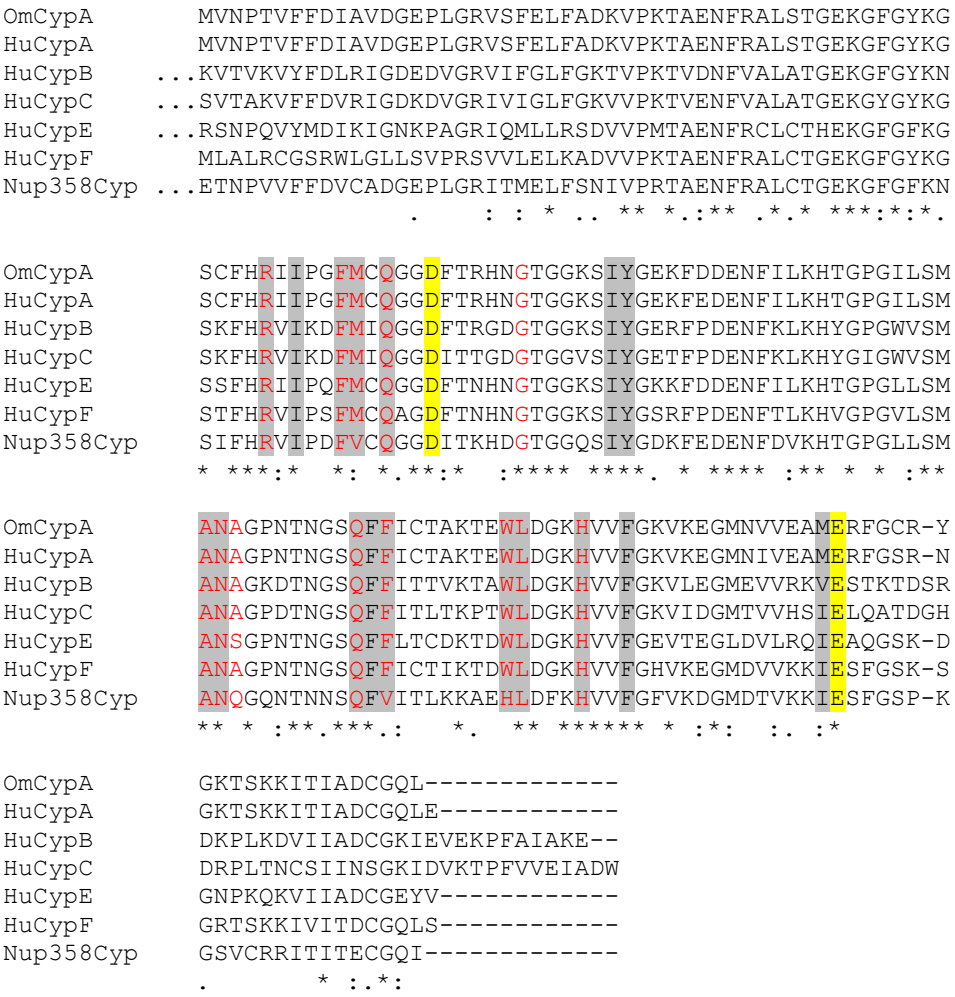

FIG 54 Amino acid alignment of catalytic domains of diverse cyclophilins

Owl monkey CypA from omTCyp was aligned with the catalytic domains of human CypA, CypB, CypC, CypE, CypF and the Cyp domain from Nup358. Amino acids highlighted in red indicate residues that are important for human CypA CsA binding [421]. Residues highlighted in grey are important for human CypA substrate binding [421]. Residues highlighted in yellow have been demonstrated to be important for specificity of macaque rhTRIMCyp for HIV-1 and HIV-2 restriction [452] (Price A. et al., unpublished). (*) identical, (:) similar or (.) weakly similar amino acids are indicated.

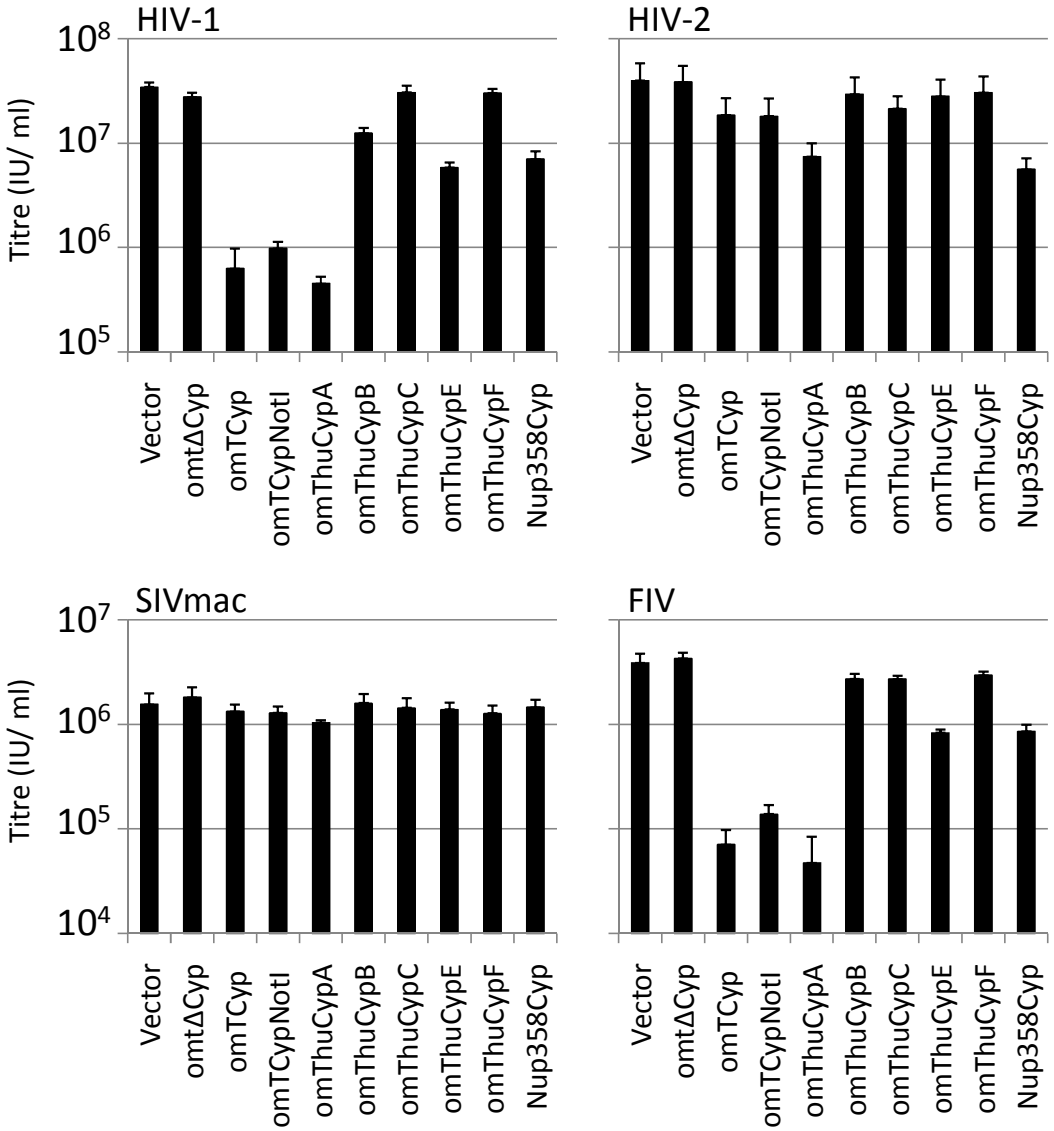

FIG 55 Restriction of diverse lentiviral vectors by owl monkey TRIMCyp fusion proteins

CRFK cells were transduced with empty EXN vector, or vectors expressing the indicated owl monkey TRIM (omT) human Cyp fusion proteins. Transduced and drug selected CRFK cell populations were infected with HIV-1, HIV-2, SIVmac or FIV GFP viral vectors and infectious titres were determined on three different virus doses. Error bars are standard deviations of mean titres determined from three different viral doses. The data are representative of two independent experiments.

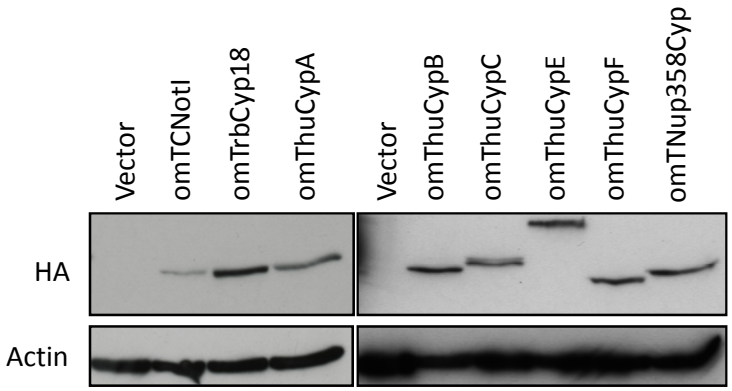

FIG 56 Measurement of omTCyp fusion protein expression levels by western blotting

CRFK cells transduced with empty EXN vector (Vector) or EXN encoding owl monkey TRIMCyp with an introduced *Not*I site, or owl monkey TRIM5 fusion proteins with human CypA, CypB, CypC, CypE, CypF and Nup358Cyp, or rabbit Cyp18 (rbCyp18) were lysed in 2 × Laemmli buffer and subjected to western blotting to detect the N-terminal HA tag. As a loading control the blot was stripped and re-probed to detect beta-actin.

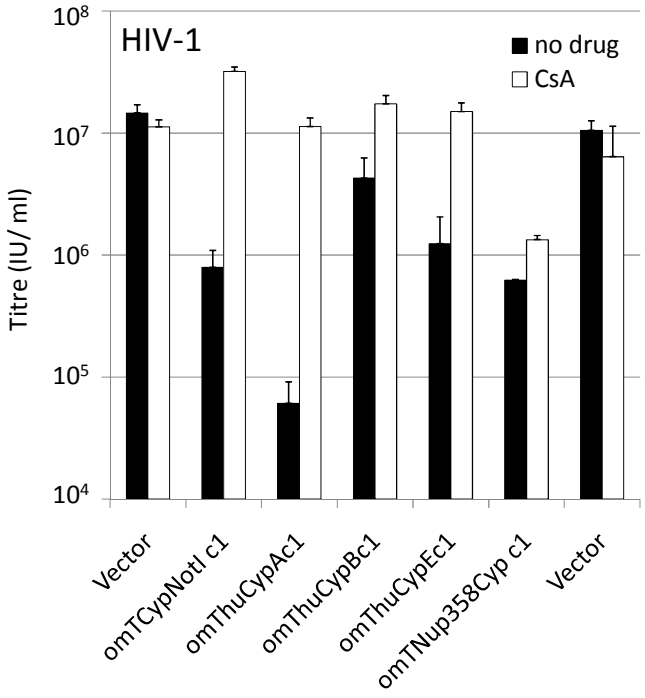

FIG 57 Infection of CRFK cell clones expressing Cyp fusion proteins with HIV-1

CRFK cell clones expressing empty EXN vector or vector encoding for omTCypNotI, omThuCypA, omThuCypB, omThuCypE or omTNup358Cyp were infected with HIV-1 GFP viral vectors in the presence or absence of 5 µM CsA and infectious titres were determined. Error bars are standard deviations of mean titres determined from three different viral doses. The data are representative of two independent experiments.

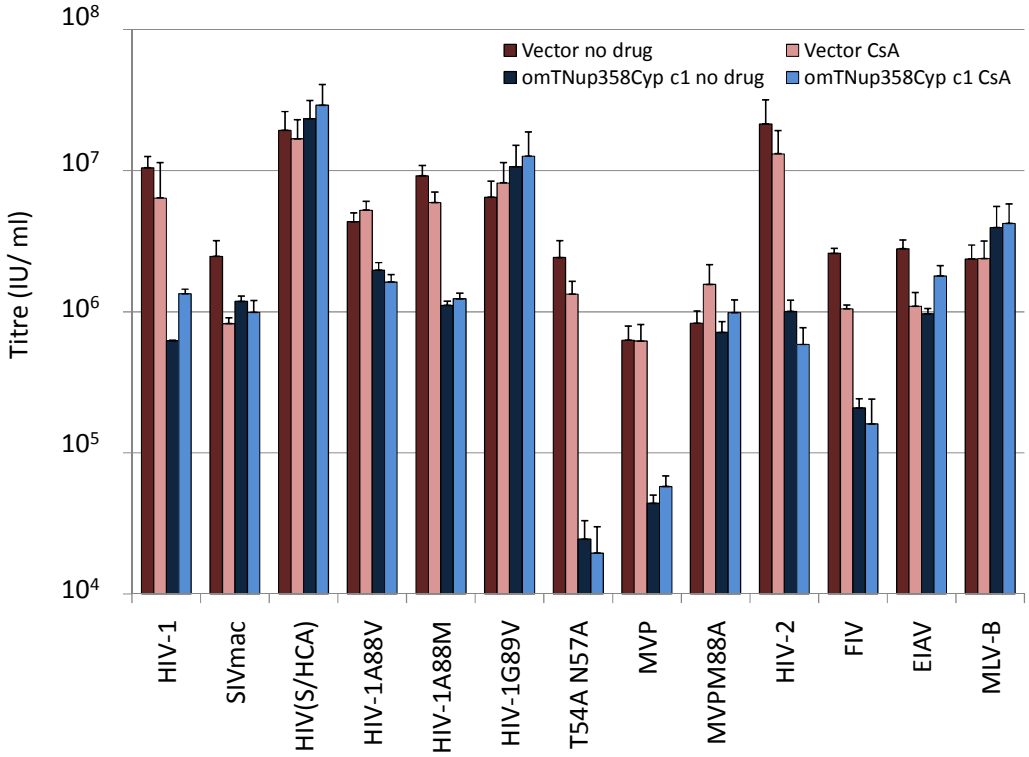

FIG 58 Infectious titres of viruses on omTNup358Cyp cells in the presence or absence of CsA

CRFK empty vector control cells or cells expressing Nup358Cyp were infected with the indicated GFP viral vectors in the presence or absence of 5 µM CsA and infectious titres were determined. Error bars are standard deviations of mean titres determined from three different viral doses. The data are representative of two independent experiments.

The implication of omTNup358Cyp binding HIV-1 CA is that CA may be involved in nuclear entry via this interaction. In order to consider this hypothesis further, I analysed the ability of omTNup358Cyp to restrict other retroviruses. CRFK cells expressing omTNup358Cyp were infected with diverse GFP encoding retroviral vectors in the presence or absence of CsA (FIG 58). M-group HIV-1 as well as O-group HIV-1 (MVP) was strongly restricted and restriction was insensitive to CsA treatment (FIG 58). Furthermore, HIV-2 and FIV were also restricted to a similar extent. In contrast, SIVmac and EIAV were only weakly (~2-fold) inhibited. HIV-1 mutant G89V as well as O-group MVP mutant MVPM88A lost sensitivity to restriction by omTNup358Cyp suggesting that like CypA, Nup358Cyp recognises the peptide bond G89-P90 in HIV-1. This is supported by the notion that the SIV-HIV-1 chimeric virus HIV(S/HCA) was also not inhibited by omTNup358Cyp. Mutation of HIV-1 CA residue A88 to M88 or V88 had no significant effect on the restriction of HIV-1. In contrast, the HIV-1 CA

double mutant T54A N57A showed markedly increased sensitivity to omTNup358Cyp compared to empty vector control cells (FIG 58). Since this mutant has been shown to be defective in infecting non-dividing cells, it is possible that Nup358 is involved in this mechanism [72].

4.2.2. The RBCC of human TRIM21 can replace the TRIM5 RBCC in TRIMCyp

The TRIM5 RBCC motif has been independently fused twice to a CypA domain by retrotransposition of a CypA cDNA close to or into the TRIM5 locus. It is not understood what advantage the TRIM5 RBCC motif has over RBCC motifs of other TRIM family members in terms of restriction. To investigate if other TRIM proteins are able to create similarly strong restriction factors the coding region of the human TRIM21 RBCC motif was used to replace the owl monkey TRIM5 RBCC in the construct omThuCypA, thereby creating a vector encoding a humanised TRIM21-CypA fusion protein. A previous study investigated the ability of RBCC domains of different human TRIM proteins, including TRIM21, to replace the owl monkey TRIMCyp RBCC domain and showed that most TRIMCyp proteins generated by fusion of the RBCC to owl monkey CypA were able to restrict HIV-1 [342]. Another study investigated fusion proteins of CypA to the RBCC domains from TRIM1, TRIM18 and TRIM19 and showed that all of these fusions were able to restrict HIV-1, however the blocks occurred at different points after virus entry [517]. Some restricted the synthesis of viral DNA and some blocked a step subsequent to nuclear import evidenced by unaltered levels of 2-LTR circles [517]. Interestingly, Li et al. reported that fusing the human TRIM21 RBCC domain to the rhesus macaque TRIM5 B30.2 domain did not create a protein that restricted HIV-1 [320]. However, replacing only the rhesus TRIM5 RING domain with that of human TRIM21, generated a chimaeric protein that was able to restrict HIV-1 potently. In comparison to wild type rhesus TRIM5α this protein had a three to four fold longer half life which could be explained by a slower proteasomal turnover [518]. In addition, wild type human TRIM21 has got a longer half life than human TRIM5α as demonstrated by western blotting experiments after cycloheximide treatment [519]. To investigate the function of the human TRIM21 RBCC motif further, it was fused to human CypA at different position and fusion proteins were tested for their ability to restrict HIV-1 infection. Different fragments of the TRIM21 RBCC coding region were cloned into omThuCypA creating EXN vectors T21CypA284,

T21CypA290 and T21CypA309 (FIG 59A). CRFK cells expressing the different TRIM21Cyps were challenged with HIV-1, or MLV-B GFP as a negative control and infectious titres were calculated. All TRIM21Cyp proteins restricted HIV-1 infection moderately when expressed in drug selected CRFK cell pools, whereas the infectivity of MLV-B was not changed, as compared to empty vector transduced cells (FIG 59B). Single cell clones were investigated for their ability to restrict HIV-1. Both, T21CypA284 and T21CypA290 potently restricted HIV-1 infection by more than three orders of magnitude, whereas T21CypA309 only restricted HIV-1 by ~10-fold, likely due to lower expression levels (FIG 59D and E). Importantly, infection could be rescued by the addition of CsA.

Recently, two opposing paper have been published by Higgs et al. and Yang et al., both investigating the activity of TRIM21 on interferon regulatory factor 3 (IRF3) induced transcription activation after pathogen stimulation. Higgs et al. provided evidence for TRIM21 mediated ubiquitination and proteasomal degradation of IRF3 to reduce the IFN-β response after infection [329]. In contrast, Yang et al. suggested a positive role for TRIM21 binding to IRF3, causing increased IRF3 mediated transcription by preventing interaction of IRF3 with the PPIase Pin1 [520]. Although these two functions seem to be opposing, it is possible that TRIM21 acts in both ways on IRF3, depending whether it occurs in an early stage during pathogen recognition or in a post-pathogen recognition situation when the cell needs to reduce the IFN response. The PRY SPRY or B30.2 domain of wild type TRIM21 can bind the Fc domain of IgG antibodies suggesting that TRIM21 may be involved in recognition of antibody opsonised pathogens [521]. The theory that TRIM21 recruits the proteasome machinery seems attractive since one could imagine that TRIM21 binding to intracellular antibody opsonised pathogens may cause their degradation via the proteasome. To examine, whether the TRIM21 RBCC can recruit the proteasomal degradation machinery, TRIM21 Cyp expressing cells were infected with HIV-1 and *GFP* RT products were measured 6 h after HIV-1 infection in the presence or absence of either, CsA or MG132. *GFP* RT products were reduced during restricted infection and could be rescued by either, CsA or MG132. Infection of HIV-1 however, could only be rescued by CsA and not MG132. This is similar to restriction by TRIM5s or TRIMCyps. The results suggest that TRIM21 can restrict HIV-1 in the same way as TRIM5 when it is fused to CypA. To investigate whether TRIM21Cyps have an antiviral activity in human cells, TE671 cells were transduced with EXN expressing T21CypA284 and the antiviral effect on

HIV-1 infection was measured in single cell clones. HIV-1 was strongly restricted (>30-fold) in TE671 cells expressing T21CypA284 and the infection could be rescued by the addition of CsA (FIG 60A). *In vitro* studies have shown that TRIM5α may be cross-ubiquitinated by TRIM21 [326]. To investigate an impact of TRIM21Cyp on endogenous TRIM5α, the TE671 cells expressing T21CypA284 were infected with MLV-N or MLV-B and infectious titres were determined (FIG 60B). Restriction of MLV-N was slightly reduced in cells expressing T21CypA284 as compared to control cells, suggesting that T21CypA284 might weakly impact on the antiviral activity of endogenous TRIM5α.

In conclusion, the results presented here show that like the TRIM5 RBCC, the TRIM21 RBCC is able to recruit the proteasome machinery since restricted HIV-1 reverse transcripts could be rescued by the inhibition of the proteasome with MG132. This suggests that TRIM21, upon target binding, causes the degradation of the recruited protein. The results suggest further that there may be a specific characteristic of TRIM5 that makes it a preferred target for the generation of a TRIMCyp fusion protein. The data presented show that TRIM21 would also make a potent CypA fusion protein restriction factor. However, so far only the TRIM5 RBCC has been found fused to CypA and this fusion has happened twice [35, 186, 187, 452].

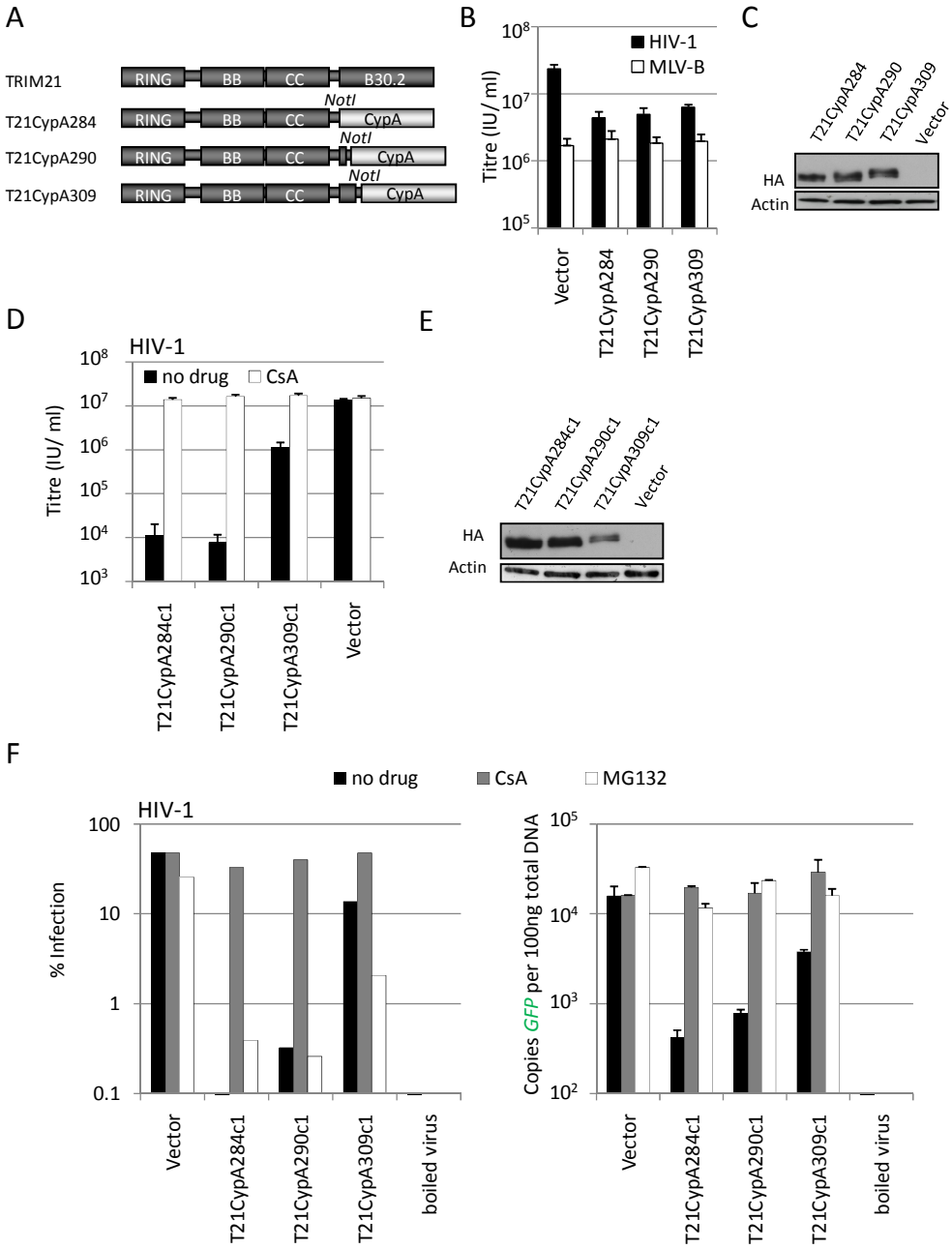

FIG 59 Analysis of TRIM21CypA fusion proteins as HIV-1 restriction factors in CRFK cells

A) Schematic representation of the TRIM21 CypA fusion constructs B) Transduced CRFK cell bulks were drug selected and infected with MLV-B or HIV-1 GFP viral vectors and infectious titres were determined. Error bars are standard deviations of the mean titres determined from three different viral doses. C) Western blotting to detect the N-terminal HA tagged fusion proteins. Beta actin was used as a loading control. D) Single cell clones were investigated on their ability to restrict HIV-1 in the presence or absence of CsA. Infectious titres and error bars determined as in B). E) HA tagged proteins from single cell clones were detected by western blotting as in C). F) Single cell clones were infected with HIV-1 GFP vectors in the presence or absence of 5 µM CsA or 8 µg/µl MG132, and percent of infected cells were determined by FACS 48 h post infection. Two in parallel infected samples were subjected to Taqman qPCR to detect *GFP* RT products 6 h post infection. *GFP* copy numbers per 100 ng extracted total DNA are plotted. As a control, samples treated with boiled viral supernatants were used. The data are representative of two independent experiments.

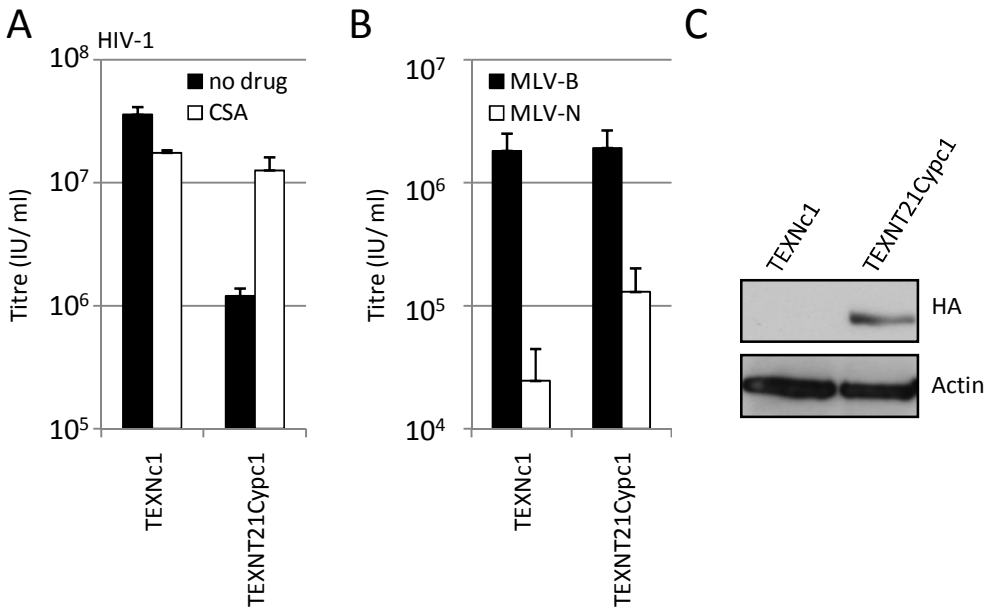

FIG 60 Analysis of TRIM21Cyp fusion proteins as HIV-1 restriction factors in TE671 cells

TE671 cells were transduced with empty EXN vector or EXN encoding for T21CypA284 and single cell clones were infected with either HIV-1 GFP vector in the presence or absence of CsA (A) or MLV-B or MLV-N GFP vectors (B) and infectious titres were determined. Error bars are standard deviations of mean titres determined from three different viral doses. C) Western blots detecting the HA tagged protein shows expression of T21CypA284. Beta actin was used as a loading control. The data are representative of two independent single cell clones analysed.

4.2.3. Fusion of CypA to Fv1N enables restriction of HIV-1 and FIV

Consideration of barriers to murine leukaemia virus (MLV) infection in mice led to the discovery of the Friend virus susceptibility gene 1 (*Fv1*) (see 1.7.). The *Fv1* gene is unique to mice and encodes an almost full-length endogenous retroviral Gag protein, which protects mice from infection by MLV.

To better understand the restriction mechanism of Fv1, fusion proteins between Fv1 and CypA were generated with an aim of expanding the specificity of restriction to non-MLV viruses. The generation of Fv1Cyp fusion proteins also provided a tool to investigate whether a natural occurring TRIMCyp fusion molecule depends on the enzymatic activity exerted by its RBCC motif to function as a restriction factor, or not. Two different Fv1CypA chimearic proteins were generated by fusing owl monkey CypA C-terminal to Fv1N amino acid residues K358 or L432 (FIG 61). To avoid disruption of dimerisation the fusion sites were chosen in the C-terminal domain [188]. In addition to a shorter C-terminus, Fv1N differs from Fv1B at amino acid residues 358 and 399 and both are important for restriction specificity [188]. In fact, it was demonstrated that position 358 had the strongest impact on the specificity of restriction.

An Fv1 protein with a glutamate at this position always restricted MLV-N, whereas a lysine at the same position changed the specificity to MLV-B [286]. Since residue K358 appears to be important in restriction by Fv1N, it was kept in the Fv1Cyp fusion proteins. Furthermore, a deletion study by Bishop et al. demonstrated that Fv1N terminating at residue 437 had full activity against MLV-B, whereas termination at residue 410 led to a loss of restriction [290].

The generated MLV vectors CFCRFv1Cyp358 and CFCRFv1Cyp432 were used to transduce CRFK cells and single cell clones were tested for their permissivity to infection with HIV-1, HIV-1 CA mutant G89V, FIV, HIV-2, MLV-B or MLV-N GFP virus like particles (FIG 62A-D and FIG 63). HIV-1 CA mutant G89V does not bind CypA [426]. Fv1Cyp358c1 and Fv1Cyp432c1 both potently restricted infection by HIV-1 and FIV by ~20-fold (FIG 62A and C). Addition of 5 µM CsA rescued the infectivity of restricted HIV-1 and FIV to levels seen in unmodified control cells. Addition of CsA in CRFK control cells had no effect on the titre of HIV-1 and slightly decreased infectivity of FIV. HIV-1G89V was equally infectious in unmodified CRFK cells, Fv1Cyp358c1 and Fv1Cyp432c1 cells, suggesting that as expected Fv1Cyp restriction occurred via binding to the HIV-1 CA in a similar way as seen for TRIMCyp (FIG 62B). Moreover, HIV-2 is not restricted by owl monkey TRIMCyp and was also not inhibited by either of the two Fv1Cyp proteins, suggesting that it is unable to bind to the owl monkey CypA domain (FIG 62D). This is in contrast to some studies that observed HIV-2 restriction by owl monkey TRIMCyp, suggesting differences depending on cell types, expression levels or the presence of the TRIM5 RBCC [452]. The addition of CsA also had no effect on the infectivity of HIV-2 (FIG 62D). Fv1Cyp was generated by fusion of CypA to Fv1N that normally restricts MLV-B in a CsA independent manner (FIG 63A). In contrast, fusing CypA to the C-terminus of Fv1N residue 358 or 432 disrupted its ability to restrict MLV-B (FIG 63A). This is in agreement with a study demonstrating that both the C- and N-terminus are important for restriction of MLV-B [290]. MLV-N was neither restricted by Fv1N nor by any of the Fv1Cyp fusion proteins (FIG 63B). The experiments were repeated on a second set of cell clones and gave similar results (data not shown).

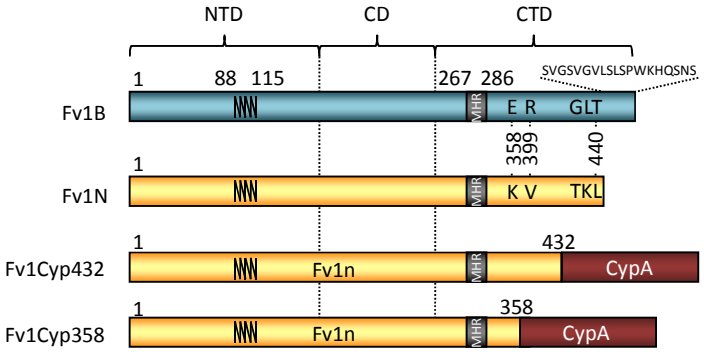

FIG 61 Schematic representation of Fv1Cyp fusion proteins

Figure shows Fv1B and Fv1N domain organization in comparison to the engineered Fv1Cyp fusion proteins. NTD, N-terminal domain; CD, central domain; CTD, C-terminal domain; MHR, major homology region. The predicted N-terminal alpha helical domain is indicated (И).

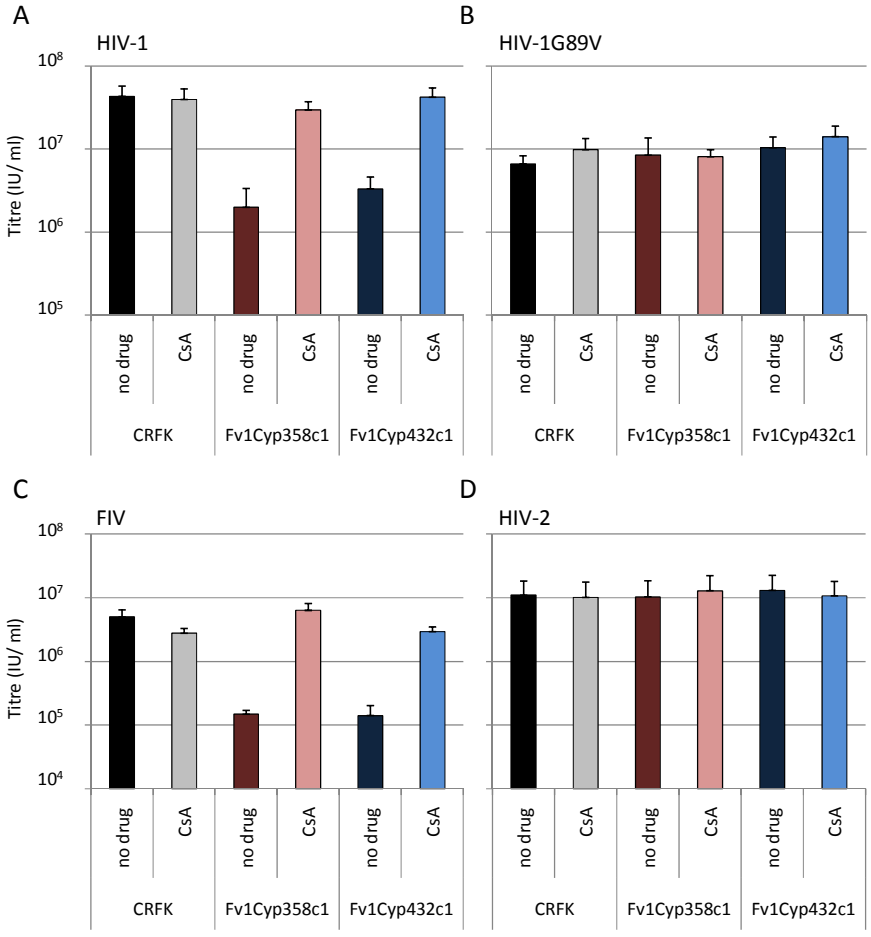

FIG 62 Analysis of Fv1Cyp restriction in CRFK cells

Unmodified CRFK cells, or cell clones expressing Fv1Cyp358 or Fv1Cyp432 were infected with HIV-1 (A), HIV-1G89V (B), FIV (C) or HIV-2 (D) GFP viral vectors in the presence or absence of 5 μM CsA. Infectious titres were determined. Error bars are standard deviations of mean titres determined from three different viral doses. The data are representative of two independent single cell clones analysed.

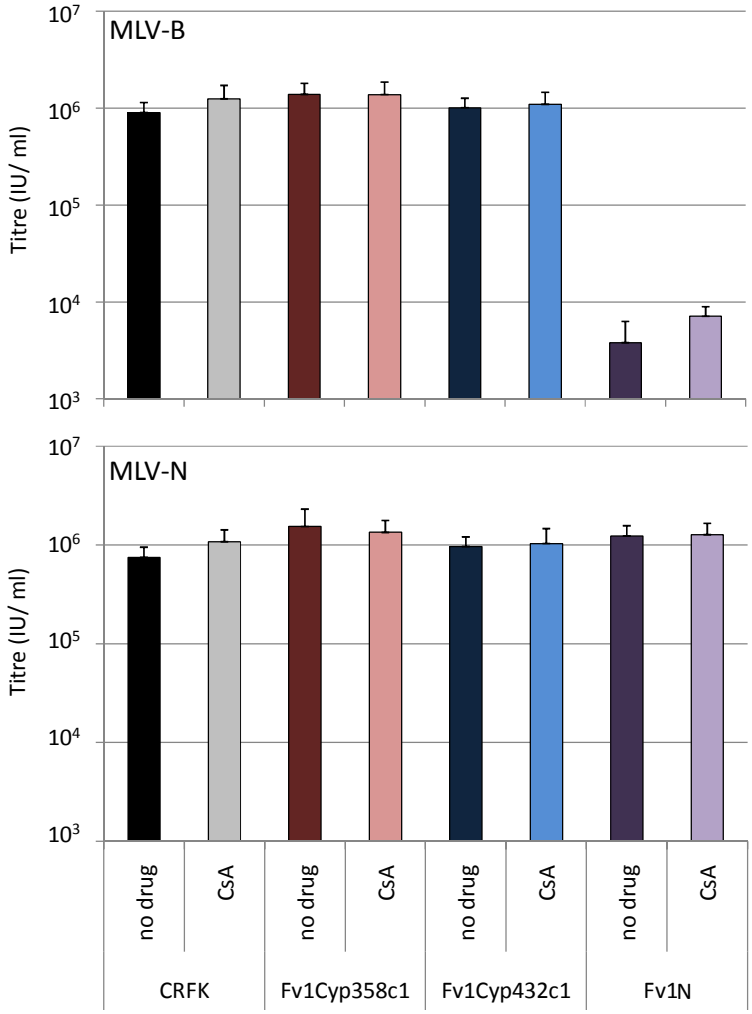

FIG 63 Infectious titres of MLV-N and MLV-B GFP vectors on Fv1Cyp expressing CRFK cells

Unmodified CRFK cells, or cell clones expressing Fv1Cyp358, Fv1Cyp432 or wild type Fv1N were infected with MLV-B (A) or MLV-N (B) GFP encoding viral vectors in the presence or absence of 5 µM CsA. Infectious titres were determined. Error bars are standard deviations of the mean titres determined from three different viral doses. The data are representative of two independent single cell clones analysed.

4.2.4. Fv1Cyp in CRFK restricts HIV-1 after 2-LTR circle formation

Fv1 restricts MLV after reverse transcription but before viral DNA circle formation, suggesting that restriction occurs at a step before nuclear entry [288, 289]. To analyse whether restriction by Fv1Cyp affects viral DNA synthesis, qPCR analyses were performed (see 2.2.9.). To investigate the ability of restricted virus to reverse transcribe, *GFP* RT products were measured at 6 h after infection, a later RT product between the 5'*LTR* and *Gag* was measured after 12 h and 18 h and HIV-1 2-LTR DNA circles were

measured 12 and 18 h after infection using specific primers and probes (see 2.1.5.). When Fv1Cyp expressing cells were infected with an MOI of ~0.1, Fv1Cyp fusion proteins restricted the infection by ~10- fold and infectivity could be rescued by addition of 5 μM CsA (FIG 64A). Neither early RT products (*GFP*) (FIG 64B), nor late RT products (*LTR-Gag*) (FIG 64B-E) were reduced in Fv1Cyp restricted infection, suggesting that Fv1Cyp blocked HIV-1 infection but not reverse transcription. To control for DNA plasmid in supernatants carried over from the 293T transfection, cells inoculated with boiled viral supernatant were analysed (FIG 64B, E and G). Moreover, 2-LTR circles were also not decreased in Fv1Cyp restricted virus (FIG 64F, G and I). In initial experiments using 2-LTR primers and probe described in [113] an MOI of >1 had to be used to detect 2-LTR circles (FIG 64H and I). When infected at this MOI, Fv1Cyp restricted HIV-1 infection resulted in increased 2-LTR circle levels that decreased upon rescue of infection by addition of CsA (FIG 64I). Increased levels of 2-LTR circles resemble a phenotype seen for integrase mutants that are unable to integrate, suggesting that the block seen for Fv1Cyps occurs after nuclear entry but before integration.

2-LTR circles were also measured by a more sensitive qPCR assay [476] in the same samples that had been used to measure *LRT-P* at 12h and 18h post infection (FIG 64F and G). In agreement with results obtained using an MOI>1, at an MOI of 0.1 no block to 2-LTR circles was detected for Fv1Cyp restricted HIV-1 (FIG 64F and G).

The results indicate that Fv1Cyp restriction is mechanistically different to restriction of HIV-1 by owl monkey TRIMCyp. Whereas owl monkey TRIMCyp restricts infection before significant viral DNA levels can be generated by RT, Fv1Cyp expression in CRFK cells results in HIV-1 restriction after RT and even after 2-LTR circle formation. In this way Fv1Cyp restriction also differs from MLV restriction by Fv1 in which circularised viral DNA forms are blocked but linear DNA is not [288]. The data suggest that Fv1Cyp forms a complex with incoming HIV-1, which is able to enter the nucleus but is unable to integrate.

Interestingly, a study investigating CypA fusion proteins with different TRIMs revealed similar results showing that some TRIM-CypA fusions did not restrict 2-LTR circles, also suggesting that PICs with bound restriction factors are able to enter the nucleus [517]. The reason why some TRIM-CypA fusions restrict at a late step and some restrict infection very early, before reverse transcription can occur, is unknown but may be explained by differences in their RBCC driven proteasomal turnover.

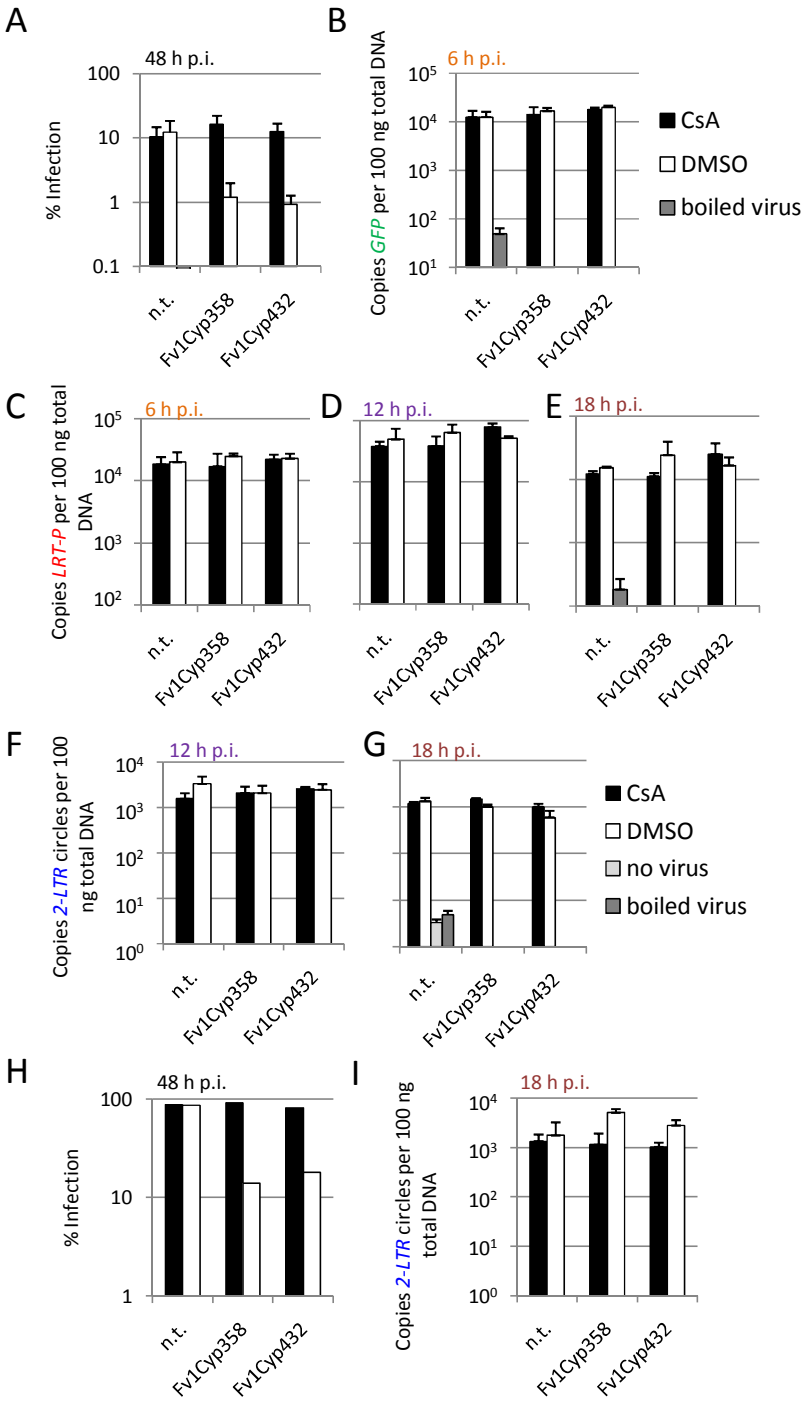

FIG 64 Taqman qPCR to measure *GFP*, *LRT-P* and 2-LTR circles for Fv1Cyp restricted HIV-1

Unmodified CRFK cells (n.t.), or cells expressing Fv1Cyp358c1 or Fv1Cyp432c1 were infected with HIV-1 GFP vector at an MOI of ~0.1 (A-G) or >1 (H, I) and *GFP* (B), *LRT-P* (C-E) viral DNA RT products or 2-LTR circles (F-I) were measured by qPCR at the indicated time points post infection (p.i.). 2-LTR primers/probe set were derived from [476] (F, G), and from [113] (E, I), respectively. Parallel samples were used to determine infected cell numbers 48 h p.i. (A and H). Infections were performed in the presence of 5 µM CsA or DMSO. As controls for plasmid contamination samples inoculated with boiled viral supernatant were analysed. Error bars are standard deviations of mean amplicon numbers per 100 ng total DNA of two parallel infections. The data are representative of two independent experiments.

4.2.5. Infectivity of Fv1Cyp restricted HIV-1 can be rescued for long time

TRIMCyp is rapidly turned over by the proteasome and HIV-1 infection of virus restricted by TRIMCyp can only be rescued by addition of CsA for a very short time period, approximately 1h after infection [522]. The observation that Fv1Cyp proteins in CRFK cells restricted HIV-1 at a very late step after RT and even after 2-LTR DNA circle formation raised the question of how long after infection restricted HIV-1 could be rescued by CsA. To analyse this, CRFK cells expressing owl monkey TRIMCyp, Fv1Cyp358 or Fv1Cyp432 were inoculated with VSV-G pseudotyped HIV-1 or MLV-B GFP vectors and virus binding was allowed by incubation on ice for 1 h. Virus infection was started by the addition of warm media and left for 10 min to generate a pulse of infection. Infection was then stopped by the addition of 40 mM ammonium chloride before starting the time course of CsA rescue (see 2.2.16.). The data show that MLV-B infection of cells expressing TRIMCyp, Fv1Cyp358 or Fv1Cyp432 was not changed by CsA (FIG 65A, B and C). In contrast, adding CsA at the start of the time course rescued HIV-1 infection in TRIMCyp expressing cells by >100-fold (FIG 65A) and in Fv1Cyp expressing cells by 30 to 60-fold (FIG 65B and C). Following the time course of CsA addition the data show that TRIMCyp restricted HIV-1 could only be rescued for up to 1h after inoculation of the virus, in agreement with previous work [522]. In contrast, Fv1Cyp restricted viral infection could be rescued by ~5-fold in case of Fv1Cyp432c1 and >20-fold in case of Fv1Cyp358c1 for up to 10 h, indicating that the virus-Fv1Cyp complex is very stable and can be readily disrupted by CsA addition at late times after inoculation, allowing infection. Since in CRFK cells 2-LTR circles were not blocked by Fv1Cyp assuming nuclear entry of the restriction complex, it seems plausible that the late rescue occurs by disrupting Fv1Cyp/HIV complexes in or at the nucleus.

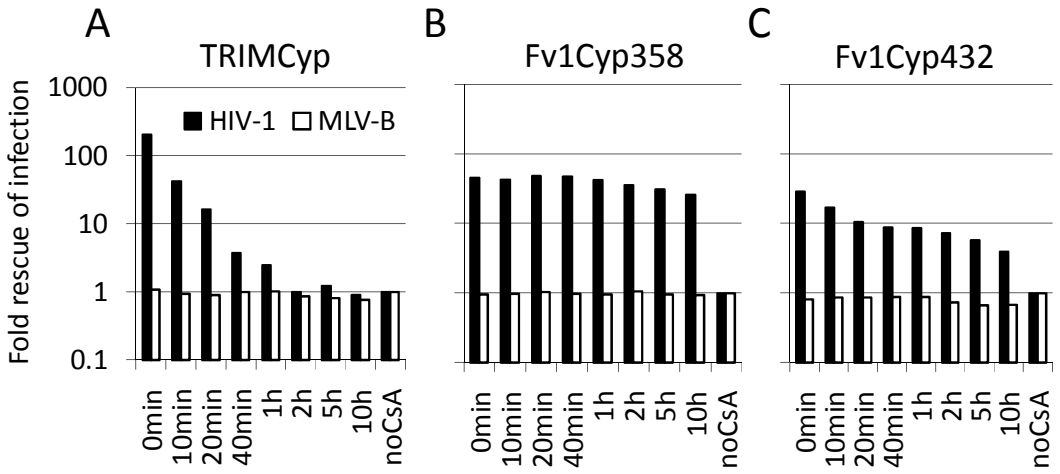

FIG 65 Time course of CsA addition in CRFK cells expressing Fv1Cyp

CRFK cells expressing A) TRIMCyp, B) Fv1Cyp358or C) Fv1Cyp432 were pulsed with a single dose of HIV-1 or MLV-B GFP viral vectors and infectivity was measured after the addition of CsA over a time course of 10 h. The graphs show the fold rescue of infectivity normalised to infection without CsA for both viral vectors. The data are representative of two independent experiments.

4.2.6. Fv1Cyp is a dominant negative factor against Fv1N restriction of MLV-B

Dimerisation is a common feature of intracellular restriction factors and both Fv1 and TRIM5α have been shown to dimerise [184, 291]. To examine whether Fv1Cyp can dimerise with Fv1N, I expressed Fv1Cyp358 and Fv1Cyp432 in cells already producing Fv1N [293]. Doubly-transduced cells were then challenged with MLV-N and MLV-B GFP viral vectors and infectivities were measured. Interestingly, expression of Fv1Cyp432 but not Fv1Cyp358 efficiently rescued infectivity of Fv1N restricted MLV-B and did not affect MLV-N (FIG 66A and B). This suggests that Fv1Cyp432 but not Fv1Cyp358 dimerises with Fv1N. To compare the transduction efficiency, the Fv1Cyp358 and Fv1Cyp432 delivering MLV dsRed vectors were titrated onto unmodified CRFK cells and infectious titres were calculated. The MLV vector delivering Fv1Cyp358 was ~6-fold higher concentrated as compared to Fv1Cyp432 delivering vector (FIG 66C). This suggests that the differences observed in the dominant negative activity of Fv1Cyp358 and Fv1Cyp432 against Fv1N in doubly-transduced cells was not due to different virus doses.

Similar experiments were performed using CRFK cells expressing HA tagged Fv1N from the MLV vector EXN. HAFv1N expressing cell populations were infected with

similar doses of MLV-N or MLV-B GFP vectors. MLV-B was blocked in HAFv1N expressing cells by ~8-fold as compared to MLV-N (FIG 66D). Transient transduction of these cells with MLV vector encoding Fv1N further decreased the infectivity of MLV-B by ~10-fold, whereas MLV-N infectivity was not changed (FIG 66D). Expression of HA tagged Fv1Cyp432 in HAFv1N transduced cells specifically increased MLV-B, but not MLV-N infectivity by ~8-fold (FIG 66D). Fv1Cyp432 transduced cells were infected in parallel with HIV-1 or FIV GFP vectors and infectivities were measured. The data show that both viruses were restricted by ~8-fold after expression of HAFv1Cyp432 (FIG 66D). This suggests that Fv1Cyp432 formed dimers with HAFv1N, which relieved restriction of MLV-B. In addition it was able to potently restrict HIV-1 and FIV. The results presented here suggest that dimerisation is critical for efficient Fv1N restriction of MLV-B. This is in agreement with studies demonstrating dominant negative effects of Fv1 mutants against Fv1N, rescuing MLV-B infectivity [286].

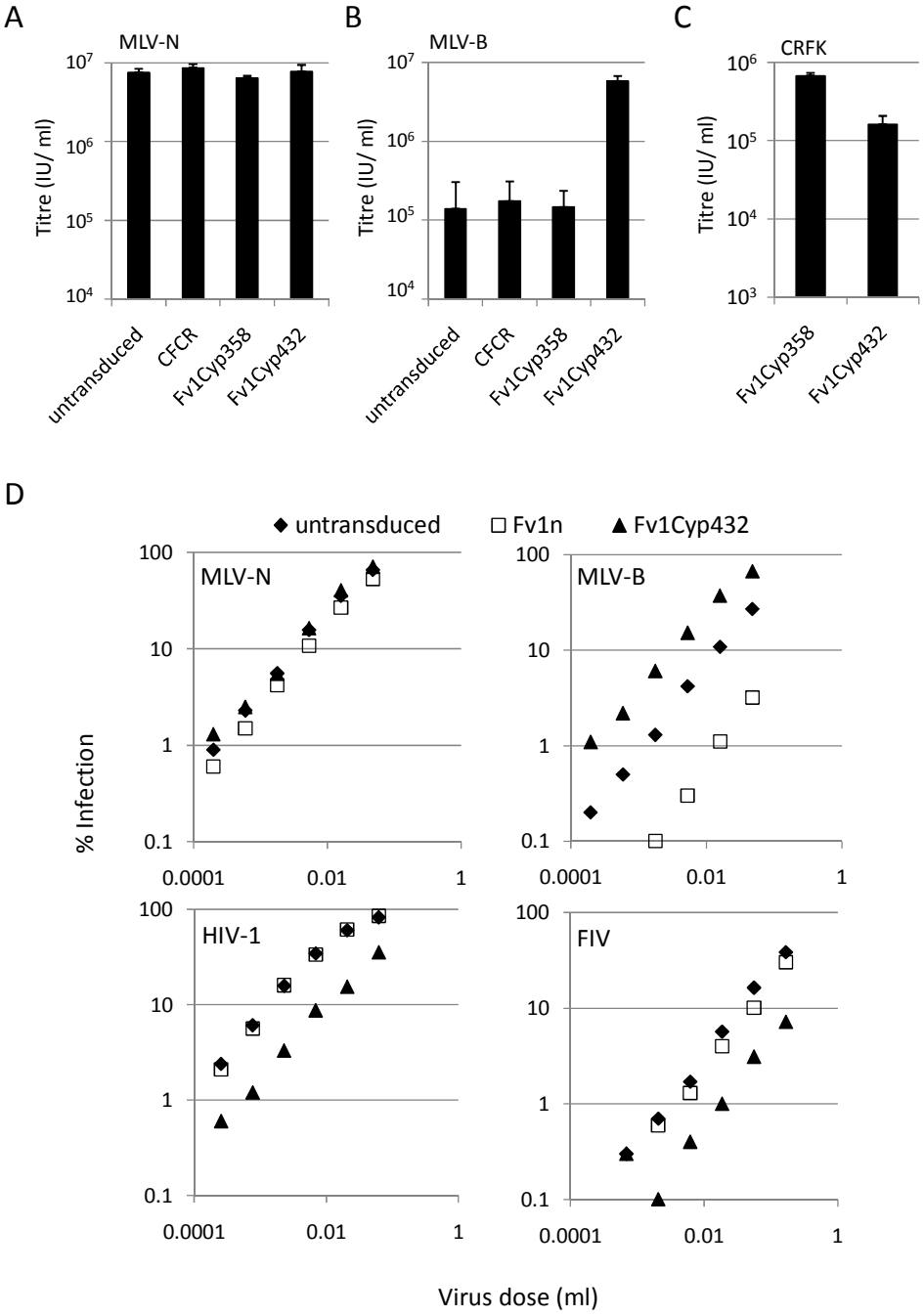

FIG 66 Fv1Cyp432 acts as a dominant negative against Fv1N restriction of MLV-B

CRFK cells expressing Fv1N [293] were transduced with vector encoding Fv1N (CFCR), or vector encoding Fv1Cyp358 or Fv1Cyp432, or left untransduced. Transduced cells were then infected with MLV-N (A) or MLV-B (B) GFP viral vectors and infectious titres were determined. Error bars are standard deviations of titre means determined from three different viral doses. C) CFCR_Fv1Cyp358 and CFCR_Fv1Cyp432 infectious titres were compared on CRFK cells. D) CRFK cell populations stably expressing HAFv1N were transduced with EXN expressing Fv1N or Fv1Cyp432, or left untreated and subsequently infected with MLV-N, MLV-B, HIV-1 or FIV GFP vectors. Infectious titres were determined 48 h post infection by FACS. Percent infection was plotted against the GFP vector dose delivered. The data are representative of two independent experiments.

4.3. Discussion

The data presented show that the cyclophilin A (CypA) domain in owl monkey TRIMCyp (omTCyp) can be replaced with a human CypA cDNA without losing the ability to restrict HIV-1 or FIV. This is not surprising given that the amino acid sequence of human CypA is very similar to owl monkey CypA and that monomeric human CypA binds to HIV-1 capsids after infection of target cells [435]. This observation could be important for the design of gene therapy vectors expressing humanised TRIMCyps to combat HIV-1/ AIDS. In fact, fusion of human CypA onto the owl monkey TRIM5 RBCC expanded its restriction specificity to HIV-2 as compared to wild type owl monkey TRIMCyp. Some studies reported restriction of HIV-2 by exogenously expressed omTCyp [452]. In contrast, despite efficient restriction of HIV-1 and FIV by omTCyp, HIV-2 infectivity was only very weakly blocked in the experiments performed herein, which is in agreement with other published work [186]. The observation that omThuCypA was able to block HIV-2 is in agreement with recent results showing weak binding of human CypA monomers to HIV-2 capsids (Leo James, unpublished). In addition, HIV-2 has been suggested to incorporate small amounts of human CypA into virus particle, suggesting weak interaction with human CypA [523]. Interestingly, CypE and the Nup358 Cyp domain also were able to replace CypA in omTCyp, thereby creating restriction factors that blocked infectivities of diverse viruses. This suggests that CypE and Nup358 can also bind to lentiviral capsids. Although no studies have been published on the ability of CypE to interact with HIV-1 capsids, the fact that it is closely related to human CypA (FIG 54) is likely to explain this observation. However, restriction of omThuCypE never reached the levels of restriction by omTCyp or omThuCypA, possibly due the extra N-terminal RNA-binding domain found in CypE. Like CypA, CypE is cytoplasmic, suggesting that it may encounter HIV-1 during infection. It would be interesting to analyse, whether CypE is also found in HIV-1 virions and whether the RNA binding domain is able to associate with HIV-1 genomic RNAs. However, the fact that omTCypE inhibition of HIV-1 infection could be abrogated by the addition of CsA suggests that CypE has no effect on virus assembly and RNA packaging since CsA treatment of virus producing cells does not significantly alter infectivity of produced HIV-1 virions [435]. OmTCypE inhibited infection by HIV-1 and weakly by FIV, whereas other tested viruses were not restricted.

Given the strong conservation to human CypA, it is very likely that the interaction of CypE occurred with the viral capsid, although this was not directly tested.

Another interesting Cyp domain that was found to interact with HIV-1 during early infection in the present study is the Nup358 Cyp domain. OmTNup358Cyp restricted HIV-1, HIV-2, FIV and O-group HIV-1. It is likely that these restrictions occurred by binding via the Nup358 Cyp domain to the viral capsids, although this was not directly tested. However, mutation of an HIV-1 CA residue known to be essential for CypA binding (G89V) also abolished restriction by omTNup358Cyp. Interestingly, the HIV-1 CA mutant T54A N57A that had been described to be impaired for infection of non-dividing cells, showed the strongest inhibition by omTNup358Cyp, which suggests that it might bind more tightly to Nup358Cyp [72]. In contrast to CypA, CypB and CypE, omtNup358Cyp restriction was insensitive to treatment with CsA likely due to differences in amino acid residues involved in drug binding (FIG 54). The data suggest that the Cyp domain of Nup358, which is probably facing the cytosol [513], may interact with viral capsids, and possibly facilitates uncoating or nuclear entry or both. This is reminiscent to the nuclear import of herpes virus genomic DNA, which is thought to occur through the nuclear pore complex. Moreover, recent studies suggest that Nup358 binds herpes simplex virus tegument proteins VP1/2 and that binding is important for nuclear import of the DNA genome [524]. The Cyp domain of Nup358 is also able to recruit proteasomal subunits, suggesting that in addition to virus interaction it may recruit the proteasome to enhance virus infection [525, 526]. A role of Nup358 in HIV-1 infection is supported by recent siRNA screens, suggesting that it may be involved in nuclear import of HIV-1 PICs [105, 107]. Braaten et al. published that a Nup358Cyp GST fusion protein was unable to bind HIV-1 Gag [400]. In contrast, the data presented here suggests that Nup358Cyp is able to bind diverse retroviral capsids when it is expressed in the context of an owl monkey TRIM fusion protein. Studies to directly investigate Nup358Cyp binding to recombinant CA-NC complexes by isothermal titration calorimetry are under way. Although the proposed substrate binding residues of human CypA are conserved in human CypC and human CypF [421], they were not able to replace the CypA domain of omTCyp. The reasons for this are unclear. The data demonstrate that CypA has an advantage over other Cyps when it is expressed as a fusion protein with owl monkey TRIM5. This is likely due to a higher binding affinity to capsid structures. However, other Cyps may serve as efficient binding domains against viruses that were not tested in this study. CypB, CypE and the

Nup358Cyp domain have the potential to bind retroviral capsid structures and may therefore serve as possible binding domains to recruit proteins to viruses. In this respect is noteworthy that CypB has been demonstrated to bind to HCV NS5B polymerase [527]. Moreover, both, CypA and CypB, have been retrotransposed several times in the human genome creating multiple pseudogenes. It is therefore possible that as yet unidentified cyclophilin fusion proteins exist which might impact on virus replication.

The investigation of artificial TRIM21 CypA fusion proteins demonstrated that the RBCC domain of TRIM21 is able to replace the TRIM5 RBCC in TRIMCyp. This is in agreement with other work, demonstrating that RBCC motifs from diverse TRIM proteins can create potent HIV-1 restriction factors when fused to CypA. Human cells that highly expressed TRIM21Cyp showed weaker restriction of MLV-N, suggesting that TRIM21Cyp may function as a dominant negative against endogenous TRIM5α. The decrease of endogenous human TRIM5α antiviral activity upon TRIM21Cyp expression might be explained by inactive heterodimer formation [341]. Alternatively, high levels of TRIM21Cyp may cross-ubiquitinate endogenous TRIM5α [326] leading to decreased TRIM5α levels and thus reduced MLV-N restriction. The data here show that TRIM21Cyp potently restricts HIV-1 and also demonstrate that the RBCC motif of TRIM21 recruits the proteasome. The data therefore supports the work by Higgs et al., showing that TRIM21 is able to mediate ubiquitination and proteasomal degradation of IRF3 [329].

Consideration of the fact that CypA fusion proteins with RBCC motifs of different TRIM proteins restrict HIV-1 at different stages during infection raised the question whether proteasomal turnover is required for a CypA fusion protein to function as a restriction factor. To investigate this, the unrelated protein Fv1 was fused to CypA and the fusion proteins were analysed for their ability to restrict different retroviruses.

Analysis of two different Fv1N CypA fusion proteins showed that they are able to inhibit HIV-1 and FIV infection in a CsA dependent way but have lost the ability to restrict MLV-B. Moreover, despite the ability of both Fv1Cyp proteins to restrict HIV-1 infection, only the longer form Fv1Cyp432 was able to saturate Fv1N restriction of MLV-B. The simplest explanation for this observation is that Fv1Cyp358 did not form heterodimers with Fv1N. This suggests that in addition to the N-terminal dimerisation domain in Fv1, C-terminal regions may be important for dimerisation [290, 291]. The present results of HIV-1 restriction by Fv1Cyp are reminiscent of studies by Yap et al. and Javanbakht et al. demonstrating that multimerisation of CypA is sufficient for

restriction of HIV-1 [528, 529]. Fv1 does not possess a RBCC motif, suggesting that it is not turned over by the proteasome by the same mechanism as TRIM5α. Moreover, Fv1N restriction of MLV occurs after the synthesis of viral DNA, but before nuclear entry and 2-LTR circle formation [288, 289]. Similarly Fv1Cyp restricts HIV-1 infection at a late stage, allowing viral DNA synthesis and even 2-LTR circle formation. 2-LTR circle formation is believed to be a marker for nuclear entry and is mediated by host cell enzymes of the non-homologous end joining pathway, in particular Ku70 and Ku80 [76]. The ability of Fv1Cyp restricted HIV-1, to generate 2-LTR circles in CRFK cells, suggests that Fv1Cyp viral complexes gain access to the nucleus. However, it is not clear how these complexes traffic into the nucleus. The sensitivity of Fv1Cyp restricted HIV-1 infection to rescue by CsA addition for up to ten hours suggests that Fv1Cyp-virus complexes are stable. A similar late block to infection has been seen for CypA fusion proteins with certain TRIM RBCC motifs, including RBCCs from TRIM1, TRIM18 and TRIM19 [517]. It is likely that these CypA fusion proteins are not efficiently turned over by the proteasome and that complexes with viral PICs are imported into the nucleus in a similar way to Fv1Cyp. However, proteasomal turnover of these proteins and the stability of the restriction complexes have not been investigated by the authors. The nuclear permeability barrier prevents energy independent exchange of molecules larger than 9 nm. However, molecules of up to 39 nm can be imported into the nucleus by an energy dependent active transport [530]. The viral capsid of HIV-1 has a diameter of ~60 nm or greater [530]. It seems unlikely that whole HIV-1 cores complexed with Fv1Cyp are imported into the nucleus through the nuclear pore. However, recent work suggests that certain HIV-1 CA residues might be involved in nuclear import, suggesting that at least some CA proteins may be present in HIV-1 PICs [72]. This CA fraction may associate with Fv1Cyp leading to nuclear import of the complex.

5. Analysis of the TRIM5/ TRIMCyp RING and B-box domains

5.1. Introduction

The mechanisms of TRIM5α and TRIMCyp restriction are unclear. TRIM5α is rapidly turned over in cells, most likely by self-ubiquitination, leading to proteasomal degradation [518]. It binds to CA proteins early after virus entry and blocks infection before significant amounts of viral DNA reverse transcription products are synthesised [184, 316]. A hypothetical model may therefore be that rapidly turned over TRIM5α dimers bind to incoming viral capsids and mediate their proteasomal degradation. Treatment of cells expressing rhesus macaque TRIM5α with proteasome inhibitors, rescues HIV-1 reverse transcription supporting the involvement of the cellular proteasome [315]. However, the notion that infection is not rescued by proteasome inhibition suggests two possibilities: 1) TRIM5α exerts its antiviral block independently of the proteasome or 2) viral capsids with bound TRIM5α are unable to orderly uncoat in the absence of the proteasome.

TRIM5α has been suggested to accelerate virus uncoating, thereby disrupting RTC and PIC formation [503]. This model has been proposed by Stremlau and colleagues who developed an assay to analyse the fate of capsid during TRIM5α mediated restriction. In this assay, lysates of TRIM5α expressing cells that were infected with restriction sensitive HIV-1 VSV-G pseudotyped vector were pelleted through 50% sucrose. Particulate capsid that pellets at the bottom of the tube is thought to resemble non-restricted virus. The loss of pellet associated CA protein in the presence of restricting TRIM5α was proposed to be the result of TRIM5α mediated disruption of viral complexes, interpreted as accelerated uncoating [503]. In addition, it was also proposed that human TRIM5α causes premature uncoating of MLV-N but not MLV-B [531]. However, how TRIM5α achieves this accelerated uncoating was not addressed by these studies. Repetition of the experiments in the presence of proteasome inhibitors demonstrated that premature uncoating of viral MLV capsids by human TRIM5α was lost upon proteasome inhibition [532]. This suggests that infectivity of restricted virus is not rescued in the presence of proteasome inhibitors due to the inability of capsids to uncoat properly upon binding by TRIM5α molecules.

As a logical consequence the restriction mechanism described as ‘accelerated uncoating’ [503] most likely resembles TRIM5α induced degradation of capsids by the

proteasome. In addition, TRIM5α levels are decreased after infection with restriction sensitive viruses in a proteasome dependent manner, whereas insensitive viruses do not decrease TRIM5α levels [533]. It is possible that blocking proteasomal degradation does not overcome the block to virus infectivity because TRIM5α mediated cross-linking of CA molecules prevents uncoating. This model implies that restriction by proteasome mediated degradation may be important for an efficient block to both reverse transcription and infection. Proteasomal degradation of TRIM5α is most likely mediated by self-ubiquitination due to the activity of the RING domain but maybe also through cross-ubiquitination by other TRIM family members (e.g. TRIM21) or unrelated ubiquitination pathways [326].

TRIMCyp shares many characteristics with TRIM5α in terms of restriction. Restriction by TRIMCyp also occurs before significant levels of DNA reverse transcription products are detected [316]. Blocking the proteasome also rescues reverse transcription and addition of CsA abolishes the block even in the presence of proteasome inhibitor [316]. However, infection is still blocked in the presence of proteasome inhibitor, again possibly due to cross-linking of CA via the CypA domains of the TRIMCyp dimer. TRIMCyp is rapidly turned over in the cell with a half life of less than 60 minutes [518]. HIV-1 restriction by owl monkey TRIMCyp occurs also rapidly, since infectivity of restricted virus can only be rescued by addition of CsA for ~1-2 h [522]. Like for TRIM5α, TRIMCyp degradation is increased in the presence of HIV-1 [533]. Furthermore, TRIMCyp dimerises similarly to TRIM5α via its coiled coil domain [343].

In this study, the function of the RING and B-box domains during TRIM5α/ TRIMCyp restriction were investigated. The hypothesised model proposes that maximal restriction of TRIM5α is achieved via RING mediated self-ubiquitination, leading to efficient proteasomal turn-over, which does not provide the virus with time to reverse transcribe. This model challenges the view, in which accelerated uncoating by TRIM5α causes the block to infection. To investigate this, RING and B-box deletion mutants as well as fusion proteins were generated and their abilities to inhibit infection as well as reverse transcription were examined.

5.2. Results

5.2.1. Fusion of the RING and B-box domains to Fv1Cyp enables it to block before RT

Analysis of Fv1Cyp restriction in CRFK cells showed that the block to HIV-1 occurs after reverse transcription of the viral RNA. Furthermore, Fv1Cyp did not affect 2-LTR circle formation, suggesting that HIV-1 Fv1Cyp restriction complexes were able to enter the nucleus (see 4.2.4.). To investigate the function of the rhesus TRIM5α RING and B-box domains in the block to HIV-1 reverse transcription, I generated constructs encoding Fv1Cyp fused to the B-box domain (BFv1Cyp) or the RING and B-box domains (RBFv1Cyp) (FIG 67). MLV vectors encoding these chimeras were used to transduce CRFK cells and drug selected single cell clones were tested for their ability to restrict HIV-1 GFP vector infection in the presence or absence of 5 µM CsA.

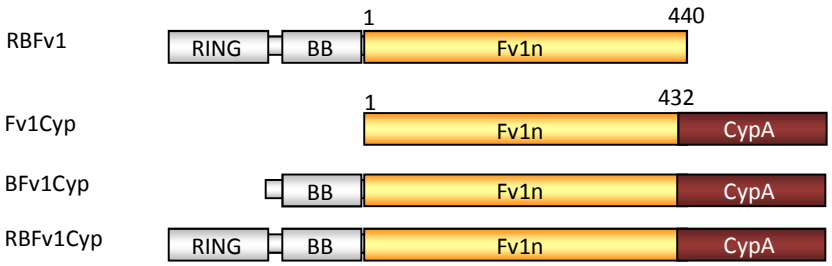

FIG 67 Schematic representation of Fv1 and generated Fv1Cyp - rhesus TRIM5 RING and B-box fusion proteins

Comparable to results obtained with untagged Fv1Cyp, HA tagged Fv1Cyp restricted HIV-1 by ~10-fold, suggesting that the HA tag does not impact on restriction (FIG 62A and FIG 68A). Fusion of the rhesus TRIM5α B-box domain to Fv1Cyp increased the block to HIV-1 slightly by two to three fold, maybe due to stronger expression levels (FIG 68B). RBFv1Cyp restricted HIV-1 infection slightly more strongly (FIG 68A). The expression levels of Fv1Cyp and RBFv1Cyp were comparable, suggesting that RBFv1Cyp did restrict HIV-1 more strongly than Fv1Cyp. Addition of CsA rescued infectivity of restricted HIV-1 in all cases up to levels seen in empty vector transduced control cells (FIG 68B).

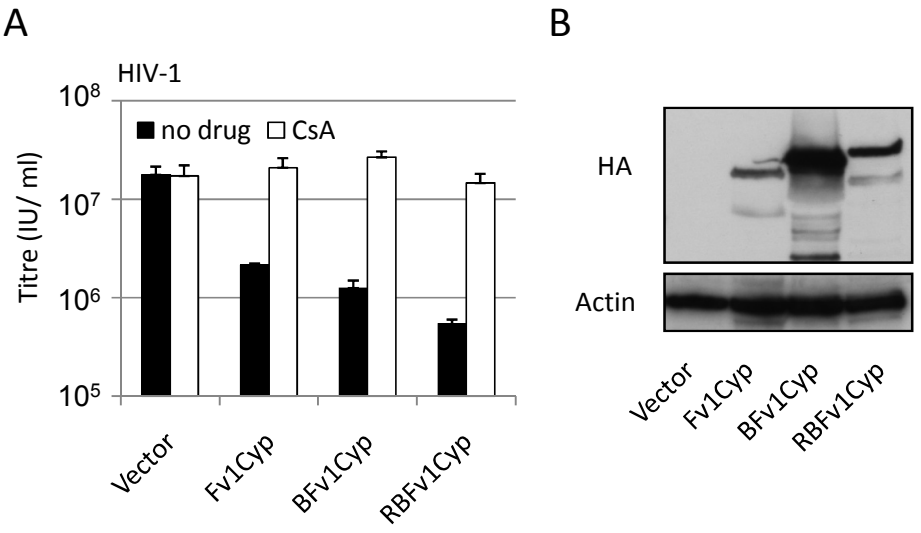

FIG 68 Investigation of the TRIM5 RING and B-box function by Fv1Cyp fusion proteins

A) CRFK cells expressing empty vector, HA tagged Fv1Cyp, BFv1Cyp or RBFv1Cyp were infected with HIV-1 GFP viral vector in the presence or absence of 5 μM CsA. Infectious titres were calculated using three different virus doses and error bars are standard deviations of mean titres determined from three different doses. B) Western blotting to detect expression levels of HA tagged proteins. Beta actin served as a loading control. The data are representative of two independent experiments.

To investigate whether BFv1Cyp and RBFv1Cyp restricted viral DNA synthesis, CRFK cells expressing the fusion proteins were infected with HIV-1 GFP vector in triplicate and *GFP* RT products in total DNA isolated 6 h after infection were measured by Taqman qPCR. As observed before, Fv1Cyp blocked infection of HIV-1 but did not inhibit the production of *GFP* RT products (FIG 69A and B). BFv1Cyp efficiently blocked HIV-1 infection (FIG 69A), but again no block against reverse transcription was observed (FIG 69B). In contrast, RBFv1Cyp efficiently blocked infection (FIG 69A) as well as the synthesis of viral DNA by reverse transcription (FIG 69B). The block to reverse transcription by RBFv1Cyp was proteasome dependent, since inhibition of the proteasome by addition of MG132 rescued *GFP* RT products to levels seen in empty vector control cells or CsA treated RBFv1Cyp cells (FIG 69B).

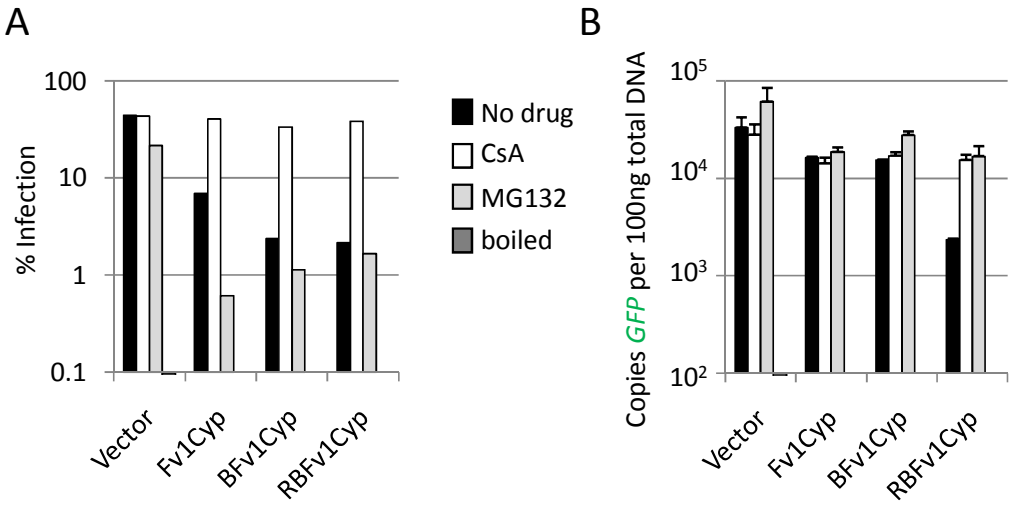

FIG 69 Fusion of the RING and B-box domains to Fv1Cyp enables a block to RT
A) CRFK cells transduced with MLV delivering empty EXN vector or vector encoding Fv1Cyp, Fv1Cyp fused to the C-terminus of the rhesus TRIM5 B-box domain (BFv1Cyp), or to the rhesus TRIM5 RING and B-box domains (RBFv1Cyp), were infected with HIV-1 GFP viral vector in the presence of 5 µM CsA, the proteasome inhibitor MG132 (8 µg/µl) or without drug and infected cells were enumerated 48 h post infection by FACS. B) Two samples infected in parallel were used to isolate total DNA which was used in Taqman qPCR to detect *GFP* RT products. Graph shows mean copy numbers of *GFP* per 100 ng total DNA. Error bars are standard deviations of mean copy numbers. The data are representative of two independent experiments.

In a similar experiment to that described above, the block to 2-LTR circle formation by Fv1Cyp, BFv1Cyp as well as RBFv1Cyp was investigated. Fv1Cyp did not block 2-LTR circles efficiently, although a moderate block to infection was observed (FIG 70A and B). In contrast, BFv1Cyp expressing cells showed a strong block to infection by HIV-1 compared to empty vector control cells (FIG 70A) and the formation of 2-LTR circles was inhibited by approximately 8-fold (FIG 70B). The observation that BFv1Cyp was unable to block early viral RT products (FIG 69), but was able to inhibit 2-LTR circle formation (FIG 70B) suggests that BFv1Cyp allowed the virus to reverse transcribe but impeded nuclear import of the RTC. RBFv1Cyp inhibited HIV-1 infection to an identical extent however 2-LTR circles were blocked more efficiently compared to BFv1Cyp (FIG 70B). The extra block in 2-LTR circles most likely reflected the observed block to early *GFP* RT products (FIG 69B).

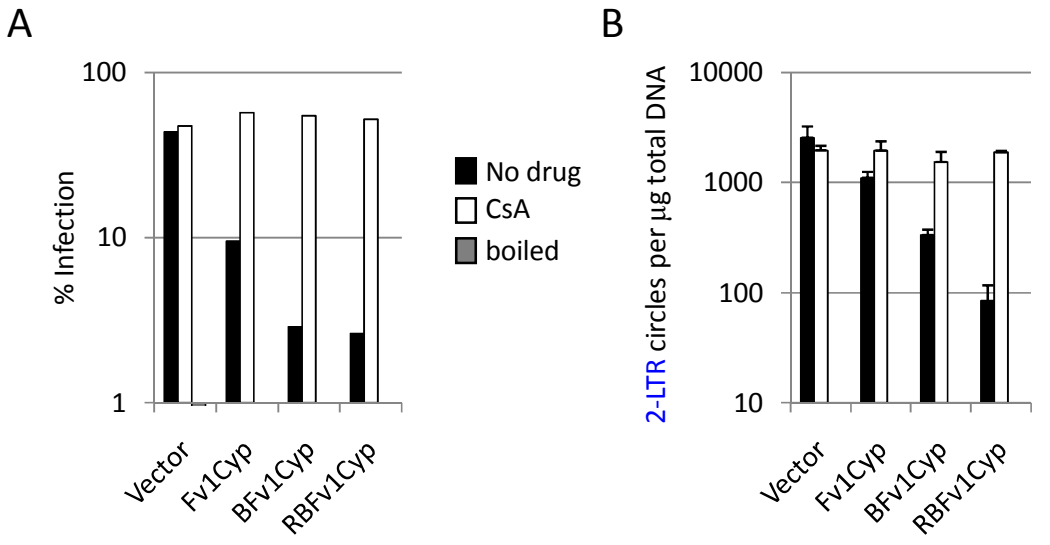

FIG 70 HIV-1 2-LTR circles are less efficiently blocked by BFv1Cyp than RBFv1Cyp

A) CRFK cell clones transduced with empty EXN vector or vector expressing Fv1Cyp, BFv1Cyp or RBFv1Cyp were infected with HIV-1 GFP viral vector and infected cells were measured 48 h post infection by FACS. B) 2-LTR circles were determined in two in parallel infected samples and mean copy numbers of 2-LTR circles per µg total DNA were calculated. Error bars are standard deviations of mean copy numbers. The data are representative of two independent experiments.

Fv1N blocks MLV-B after synthesis of viral DNA but before 2-LTR circle formation [288, 289]. In order to test whether the RING and B-box domains of rhesus TRIM5α can enable Fv1N to block MLV-B before reverse transcription, a retroviral vector encoding Fv1N fused to the RING and B-box domains was generated and used to transduce CRFK cells. Consistent with published results, Fv1N potently restricted MLV-B but not MLV-N [188]. However, fusion of the RING and B-box domains to Fv1N completely abolished the block to MLV-B infection (data not shown), despite similar levels of RBFv1N and Fv1N proteins as judged by western blotting for the HA tag (data not shown).

5.2.2. OmTRIMCyp without RING and B-box domains blocks infection but not RT

The results obtained with the Fv1Cyp and RBFv1Cyp chimaeras suggested that the RING domain is responsible for the ability of TRIM5 to block infection before early reverse transcription, whereas the B-box domain confers a block to later reverse transcription products and 2-LTR circles. To test this hypothesis directly, restriction of infection and reverse transcription were measured after expression of wild type owl

monkey TRIMCyp (omTCyp), as well as the RING deletion mutant omTCypΔ93 and the RING and B-box deletion mutant omTCypΔ128. First the ability of the mutants to restrict infection by HIV-1, HIV-2, FIV and MLV-B was investigated. Wild type omTCyp or the deletion mutants were expressed in CRFK cells and cell populations were challenged with GFP virus like particles. Wild type omTCyp as well as omTCypΔ93 efficiently blocked HIV-1 and FIV infection by almost two orders of magnitude. Owl monkey TRIMCyp with deleted RING and B-box domains still restricted HIV-1 and FIV by ~3 to 5-fold in drug selected CRFK populations. This level of restriction however is very weak as compared to the ability of the wild type protein and indicates the importance of the TRIMCyp RBCC motif, particularly the B-box domain, in restriction. In agreement with published data, MLV-B and HIV-2 were unrestricted by owl monkey TRIMCyp [186].

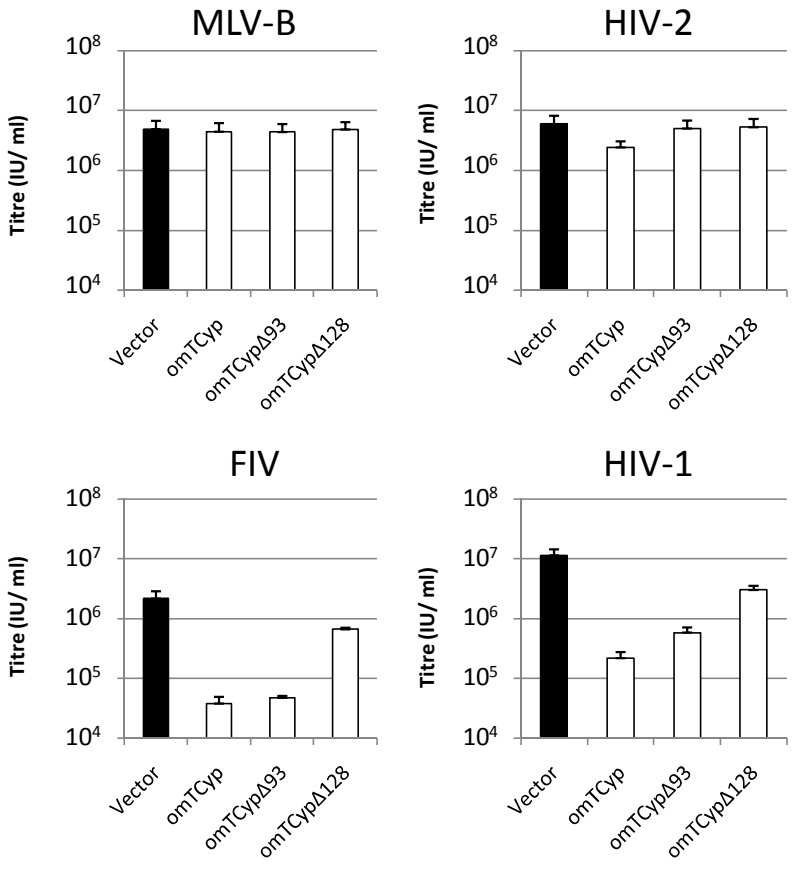

FIG 71 Restriction of owl monkey TRIMCyp deletion mutants in CRFK cells

CRFK cells were transduced with empty EXN vector, or vector expressing wild type owl monkey TRIMCyp (omTCyp), omTCypΔ93 or Δ128 and challenged with indicated GFP viral vectors. The infectious titres were determined from three different viral doses. Error bars are standard deviation of mean titres determined from three different viral doses. The data are representative of two independent experiments.

Next, the impact of the deletions on the ability of owl monkey TRIMCyp to inhibit reverse transcription of restricted virus was analysed. CRFK cell clones expressing wild type omTCyp, omTCypΔ93, or omTCypΔ128 were infected with HIV-1 GFP vector in triplicate, in the presence or absence of CsA to measure infectivity by FACS and synthesis of *GFP* RT products by Taqman qPCR (FIG 72). Wild type owl monkey TRIMCyp restricted HIV-1 infectivity by almost two orders of magnitude (FIG 72A) and the block occurred mainly before RT since *GFP* RT products were reduced by ~50-fold (FIG 72B). Deletion of the TRIMCyp RING domain (omTCypΔ93) had no effect on restriction of HIV-1 infectivity (FIG 72A). However, the block to reverse transcription was significantly reduced by almost one order of magnitude (FIG 72B). OmTCypΔ128 restricted infection by HIV-1 although the block to infection was significantly reduced compared to wild type omTCyp (FIG 72A). However, no block to reverse transcription was observed for omTCypΔ128 restricted virus and *GFP* RT products were similar to empty vector control cells or wild type omTCyp cells infected in the presence of CsA (FIG 72B). Infectivity as well as reverse transcription could be rescued for wild type omTCyp as well as for the deletion mutants by CsA addition. The obtained data was confirmed by analysing a second set of independent CRFK cell clones (data not shown).

The presented data demonstrate that homodimeric omTCypΔ93 and omTCypΔ128 both were able to restrict HIV-1 infection in a CsA sensitive way. The difference however was observed in the strength of the block. Maximal restriction of infection did not depend on the RING domain, whereas maximal restriction of reverse transcription did. Deletion of the RING never fully abolished the block to reverse transcription, suggesting a role for the B-box domain in the block to RT. Deletion of the B-box however completely ablated the block to reverse transcription, whereas virus infectivity was still restricted by ~10-fold in case of single cell clones. These data suggest that both, the RING and B-box domains have a role in blocking reverse transcription.

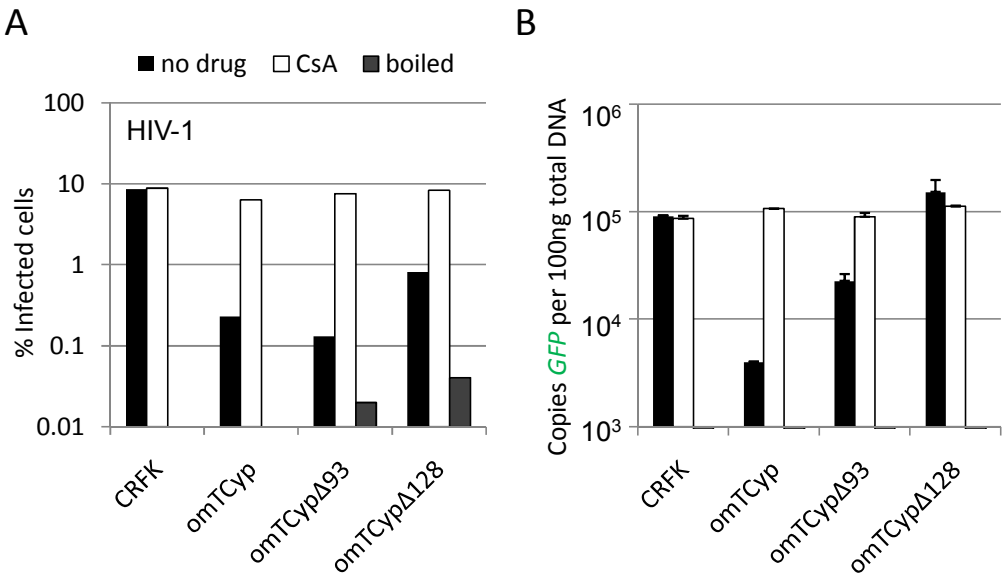

FIG 72 Owl monkey TRIMCyp deletion mutants have a decreased block to RT

A) Wild type owl monkey TRIMCyp (omTCyp), RING deleted (omTCypΔ93) or RING and B-box deleted owl monkey TRIMCyp (omTCypΔ128) were expressed in CRFK cells. Transduced cell clones were infected in parallel with HIV-1 GFP viral vector in the presence or absence of 5 µM CsA and infection was measured by FACS 48 h after infection. B) *GFP* RT products were quantified from total DNA isolated 6 h after infection by Taqman qPCR. The mean of two in parallel infected samples is shown and error bars are standard deviations of mean copy numbers. As a control for plasmid contamination cells inoculated with boiled supernatant were analysed. The experiment was repeated with an independent set of cell clones revealing similar results. The data are representative of two independent experiments.

5.2.3. Dominant negative inhibition of the block to RT but not infection in OMK cells

To test whether RING and RING-B-box deleted owl monkey TRIMCyp expression is dominant negative against the wild type protein, I expressed wild type omTCyp, omTCypΔ93, omTCypΔ128 or CypA deleted omTCyp (omTΔCyp) in owl monkey kidney (OMK) cells, already expressing endogenous TRIMCyp. These cells were then infected with HIV-1 GFP vector and infection, as well as *GFP* RT products were measured. As controls, untransduced OMK cells were infected in the presence or absence of CsA. As a control for plasmid contamination of the PCR OMK cells were inoculated with boiled viral supernatant in the presence of CsA.

Expression of omTΔCyp rescued infectivity of HIV-1 as well as reverse transcription to levels seen for cells infected in the presence of CsA (FIG 73A and B). In contrast, expression of omTCypΔ93 or omTCypΔ128 in OMK cells did not significantly rescue HIV-1 infectivity compared to empty vector control cells or untransduced cells (FIG

73A). However, expression of either truncated protein inhibited the endogenous TRIMCyp mediated block to HIV-1 reverse transcription and rescued *GFP* RT products to levels observed for control cells. This result underlines the importance of the RING domain in the block to reverse transcription observed by exogenous expression of the deletion mutants in CRFK cells (FIG 72). It suggests that for an efficient block to reverse transcription both RING domains of a TRIMCyp dimer are essential. A heterodimer of a wild type monomer and a RING deleted monomer may not be turned over as rapidly by the proteasome as a wild type homodimer, giving the virus the chance to reverse transcribe. Moreover, the dominant negative activity of omTΔCyp against endogenous TRIMCyp suggests that a monomeric CypA binding domain in a TRIMCyp dimer is insufficient for effective restriction. This is despite the fact that CypA monomers are known to bind CA monomers with high affinity and reinforces the notion that dimerisation is essential for restriction. Importantly, expression of wild type protein had no significant impact on the ability of OMK cells to restrict HIV-1 infection (FIG 73A and B).

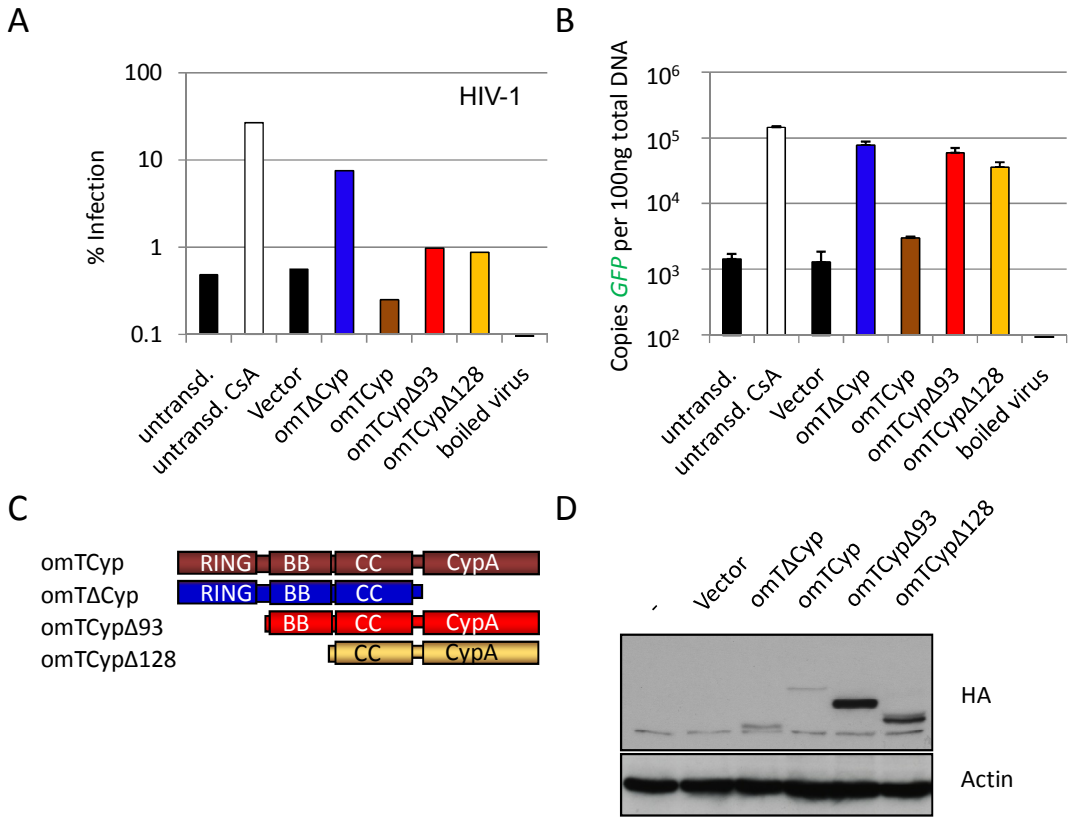

FIG 73 Expression of owl monkey TRIMCyp mutants rescues RT but not infection in OMK cells

A) Owl monkey kidney (OMK) cells were transduced with MLV delivering EXN (Vector), or EXN encoding for owl monkey TRIMCyp lacking the CypA domain (omTΔCyp), the RING domain (omTCypΔ93), or the RING and B-box domains (omTCypΔ128), or were not modified (untransd.) and infected with HIV-1 GFP viral vector in triplicate. As a positive control unmodified OMK cells were infected in the presence of 5 μM CsA. Percent infected cells were determined 48 h post infection by FACS. B) *GFP* RT products were measured by Taqman qPCR. *GFP* copy number means per 100 ng total DNA determined from two in parallel infected samples are plotted and error bars are standard deviations of the means. As a control for plasmid contamination boiled supernatant was used. C) Schematic representation of the mutants used. D) Western blotting to detect expression levels of HA tagged proteins. Beta-actin was used on stripped blots as a loading control. The data are representative of two independent experiments.

5.2.4. The block to reverse transcription by rhTRIMCyp is RING domain dependent

I also examined the role of the RING and B-box domains in virus restriction in the context of an independent naturally occurring TRIMCyp protein from rhesus macaques (FIG 74). Expression of wild type rhesus TRIMCyp (rhTCyp) in CRFK cells resulted in the restriction of FIV by ~50-fold (FIG 74A). Interestingly, addition of 5 μM CsA

during the infection did not completely rescue infectivity of the virus, which is in agreement with published data (data not shown) [452].

Deletion of the RING domain of rhesus TRIMCyp (rhTCypΔ94) had no impact on the ability of the protein to restrict FIV infection (FIG 74A). However, the block to reverse transcription was reduced. Furthermore, addition of the proteasome inhibitor MG132 rescued *GFP* reverse transcription products. This suggests that the RING domain is important for the block to reverse transcription exerted by rhTRIMCyp [452]. Similarly to owl monkey TRIMCyp, deleting both the RING and B-box domains (rhTCypΔ129) significantly impaired restriction of FIV infection and no block to reverse transcription was observed (FIG 74B). The data suggests that like for owl monkey TRIMCyp restriction of HIV-1, maximal restriction of FIV by rhesus TRIMCyp depends on the presence of the B-box domain. Deletion of the RING domain had no impact on the strength of restriction of FIV infectivity but increased RT products up to levels seen for MG132 treated cells, suggesting that the RING domain is required for the proteasome dependent block to reverse transcription.

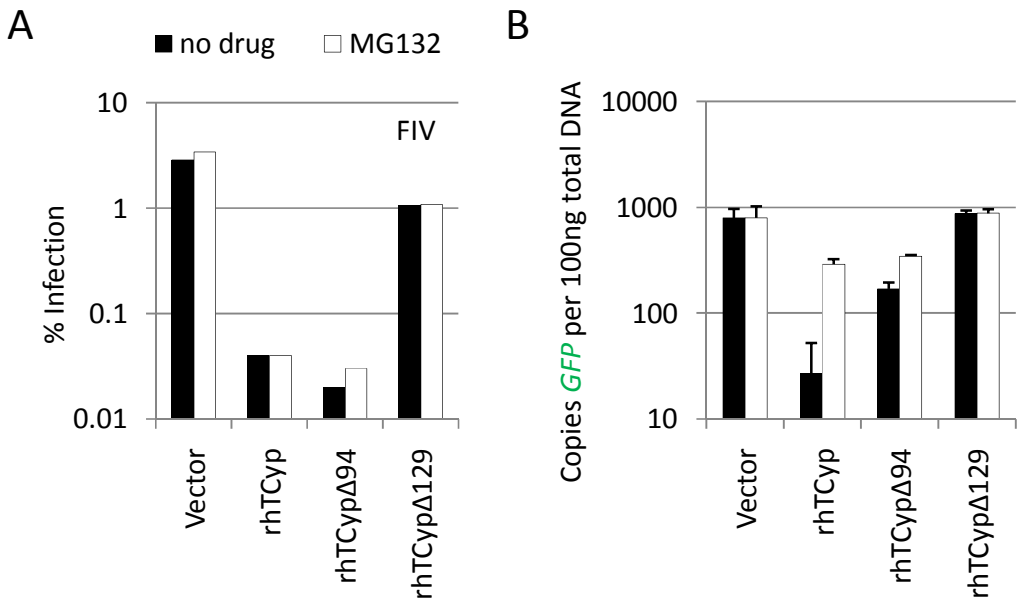

FIG 74 Rhesus TRIMCyp deletion mutants have a decreased block to RT

Wild type rhesus macaque TRIMCyp (rhTCyp), RING deleted (rhTCypΔ94) or RING and B-box deleted rhesus TRIMCyp (rhTCypΔ129) were expressed in CRFK cells. Transduced cells were infected in parallel with FIV in the presence or absence of the proteasome inhibitor MG132 and infection was measured by FACS 48h after virus inoculation (A). *GFP* RT products were quantified from total DNA isolated 6h after infection by Taqman qPCR (B). The mean of duplicate samples is shown and error bars are standard deviations. Infectivity and *GFP* copies for a boiled supernatant control on empty vector cells was below detectable limits. The data are representative of two independent experiments.

5.2.5. The requirement for the RING domain in rhTRIMCyp restriction is virus specific

Rhesus macaque TRIMCyp efficiently restricts HIV-2 and FIV, but not HIV-1 or SIVmac [186]. The experiments performed above suggested that the RING and B-box domains mediate the block to reverse transcription in TRIMCyp restriction. Owl monkey TRIMCyp restricted FIV to a similar degree as HIV-1, and restriction of either virus did not require the presence of the TRIMCyp RING domain (FIG 72). FIV restriction by rhTRIMCyp was also independent of the TRIMCyp RING domain. To test whether the requirement for the RING domain is virus specific, restriction of other rhTRIMCyp sensitive viruses was analysed.

CRFK cells expressing empty vector, wild type rhesus TRIMCyp (rhTCyp), RING deleted (rhTCypΔ94), or RING and B-box deleted rhesus TRIMCyp (rhTCypΔ129) were infected in parallel with diverse retroviral vectors indicated in FIG 75 and infectious titres were compared. In agreement with previous reports, HIV-1, SIVmac and MLV-B were insensitive to rhesus TRIMCyp (FIG 75). Interestingly, an O-group HIV-1 (MVP) was restricted by rhTCyp by ~30-fold. Deletion of the RING domain (rhTCypΔ94) had no significant impact on the restriction of HIV-1 MVP. Similarly, FIV was restricted by rhTCyp as observed before by almost three orders of magnitude and deletion of the RING had only a minor impact on the infectivity, relieving the block by ~6-fold. Restriction of MVP and FIV by rhTCyp therefore did not depend on the presence of the RING domain. In contrast, HIV-2 restriction by rhTCyp strongly depended on the RING domain since the block by wild type rhTCyp (~500-fold) was almost completely abolished by deletion of the RING in rhTCypΔ94 (~7-fold inhibition). Moreover, SIVmac mutant SIV-IG/LPA (for amino acid sequence see FIG 42), which gained sensitivity to restriction by rhTCyp, was similarly sensitive to RING deletion. Like HIV-2, SIVsm from sooty mangabeys was restricted by rhTCyp and deletion of the RING decreased the block by ~100-fold as compared to FIV (~6-fold). The HIV-2 mutant I85Q was slightly less strongly restricted by rhTCyp than HIV-2 wild type by 50-fold (FIG 75). Like for HIV-2 wild type, restriction of HIV-2I85Q by rhTCyp was strongly dependent on the presence of the RING domain and rhTCypΔ94 exerted no block to infection.

These data suggest that restriction of different viruses is differentially dependent on the presence of the RING domain and that the strength of the block does not dictate the requirement for the RING domain. The data suggest that the major block to some

viruses (HIV-2 and SIVsm) is RING dependent, whereas for other viruses (FIV, HIV-1 O-group) it is not.

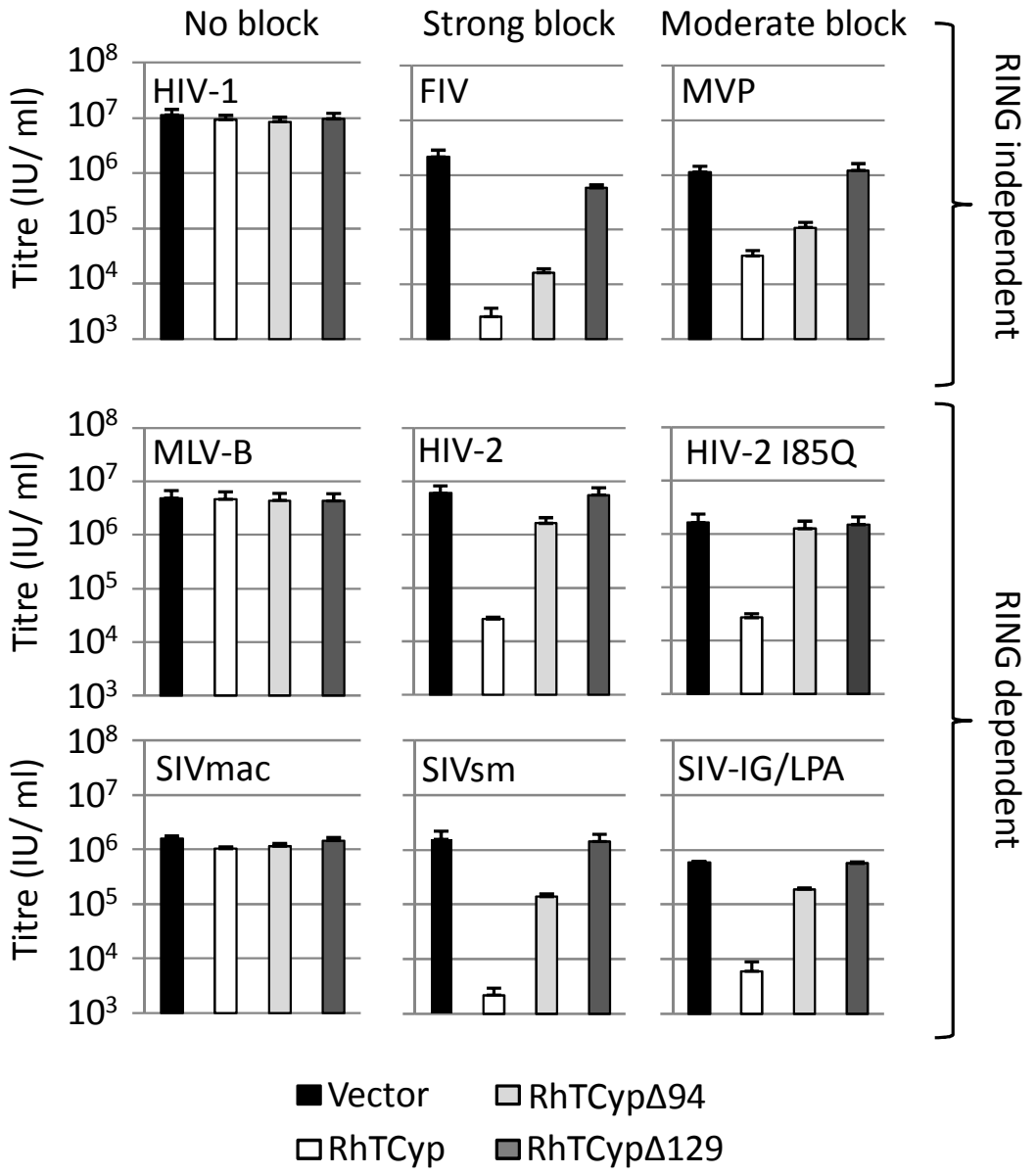

FIG 75 Differential RING domain requirements for restriction of diverse viruses by rhTRIMCyp

CRFK cells transduced with empty vector, rhTRIMCyp (rhTCyp), rhTRIMCyp with deleted RING domain (rhTCypΔ94) or deleted RING and B-box domains (rhTCypΔ129) were infected with indicated GFP viral vectors and infectious titres were determined at three different vector doses. Error bars are standard deviations of titre means determined from three different viral doses. The data are representative of two independent experiments.

5.2.6. Deletion of the human TRIM5 RING domain results in loss of MLV-N restriction

TRIMCyp restriction involves binding of the CypA domain to capsids. In contrast, TRIM5α restriction occurs via binding of the B30.2 domain. In order to determine whether the obtained results for the function of the RING and B-box domains in TRIMCyp restriction could be expanded to TRIM5α, human and rhesus macaque TRIM5α deletion mutants were generated and analysed. Furthermore, domains of human TRIM5α were substituted with domains from the heterologous human TRIM protein TRIM34, and the restriction ability was analysed and characterised.

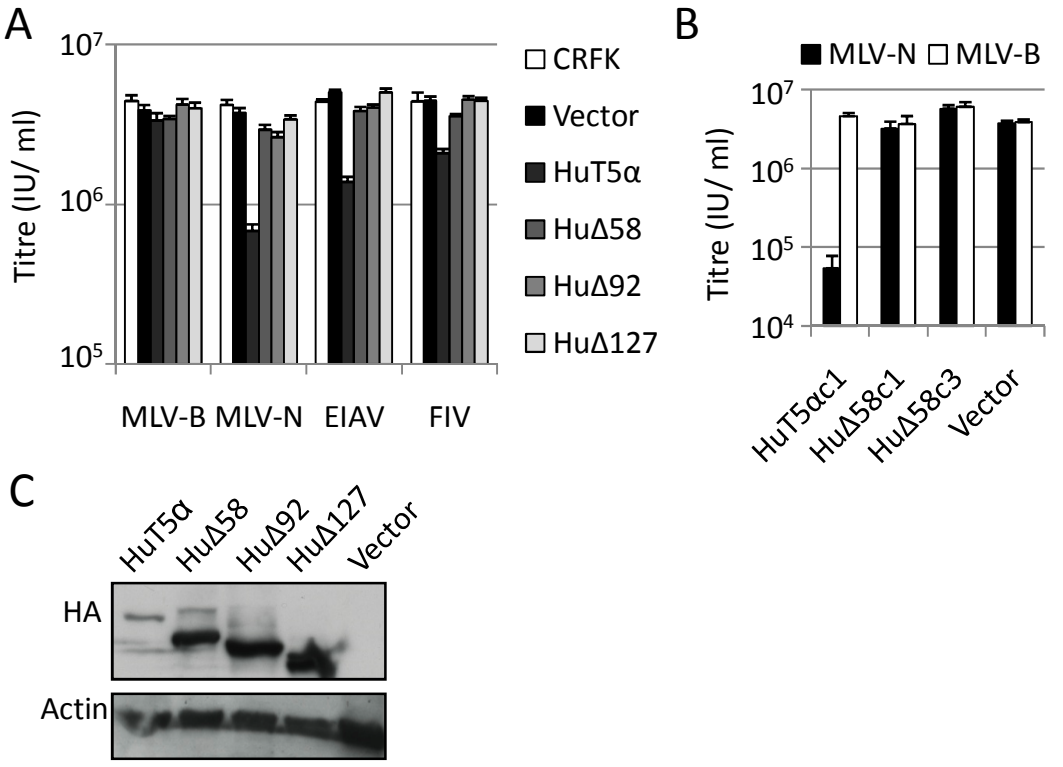

FIG 76 Analysis of human TRIM5 deletion mutants

A) CRFK cell populations transduced with empty EXN vector or vector encoding human TRIM5α, or different human TRIM5α deletion mutants were infected with VSV-G GFP vectors indicated. Untransduced CRFK cells were used as a control. Infectious titres were calculated from three different viral doses and error bars are standard deviations of the titre means. B) Single cell clones of wild type human TRIM5α, or human TRIM5α with deleted RING domain (HuΔ58) were infected with MLV-N or MLV-B GFP vectors as in A). The data are representative of two independent experiments. C) Western blotting to detect HA tagged proteins. Beta actin served as a loading control.

Human TRIM5α efficiently restricts MLV-N but not MLV-B [185]. In addition, it also moderately restricts EIAV and FIV [185, 534]. CRFK cells expressing empty EXN vector, or vector encoding for human TRIM5α, or human TRIM5α deleted in the first 58, 92 or 127 amino acids (HuΔ58, HuΔ92 or HuΔ127) were infected with MLV-N, MLV-B, FIV or EIAV and infectious titres were determined as before (FIG 76A). Wild type HuT5α expressed in CRFK cell bulks moderately restricted MLV-N and EIAV and weakly blocked FIV, whereas MLV-B was not restricted. Deletion of the first 58 amino acids completely abolished the block to restricted viruses. Further deletion (HuΔ92 and HuΔ127) also abolished restriction. The expression levels of the proteins were compared by western blotting against the N-terminal HA tag and showed that expression levels for the deletion mutants were increased compared to wild type protein levels (FIG 76C). The moderate restriction of MLV-N by wild type TRIM5α was probably a result of low expression levels. Two single CRFK cell clones of HuΔ58 were investigated and compared to a cell clone expressing wild type human TRIM5α. Neither HuΔ58c1, nor HuΔ58c3 showed restriction of MLV-N (FIG 76B). Expression levels of the two clones were comparable to wild type protein expression levels (data not shown).
A recent publication demonstrated that a single amino acid change in the human TRIM5α B30.2 domain was sufficient to extend its restriction specificity to MLV-B and it was suggested that this could be an effect of stronger association with MLV capsids [350, 532]. The mutant was tested in the background of HuΔ58 with the idea that higher affinity binding could bypass the dependency on the presence of the RING domain. However, despite strong expression levels, no block against MLV-N or MLV-B was observed (data not shown).
In agreement with published data these results show that in case of human TRIM5α restriction of MLV-N the RING domain is crucial and deletion causes the complete loss of restriction [344].

5.2.7. Replacement of the human TRIM5 RING with the TRIM34 RING domain

TRIM5α is rapidly turned over by the proteasome, whereas other TRIM proteins, including TRIM34 and TRIM21, are less quickly turned over when expressed in HeLa cells [535]. Rapid proteasomal turnover of rhesus TRIM5α however is not a requirement for HIV-1 restriction per se [518]. A chimaeric rhesus macaque TRIM5α protein with its RING domain replaced by the TRIM21 RING domain is still able to potently restrict HIV-1 infectivity however has a shorter half life as compared to wild type TRIM5α [518]. As demonstrated above, the human TRIM5α RING domain is essential for restriction of MLV-N and deletion leads to a complete loss of restriction. To address the question, whether the loss of restriction is due to a decreased turn over in the absence of the RING domain, a chimaeric protein in which the human TRIM5α RING domain was replaced by the RING domain of TRIM34 was engineered and tested for its activity against MLV-N and MLV-B (FIG 77). MLV-N restriction was significantly increased in T34R5 (1000-fold) compared to wild type human TRIM5α (50-fold) expressing cells, likely due to higher expression levels (FIG 77A and C). To test whether, like TRIM5α, T34R5 blocks MLV-N infection before reverse transcription, *GFP* copies of MLV-N or MLV-B viral vector infected cells expressing human TRIM5α or T34R5 were measured and compared. Despite a stronger block to infection, levels of *GFP* RT product were slightly increased in T34R5 expressing cells, suggesting that the protein was less efficiently degraded by the proteasome (FIG 77B). However, there was still a 10-fold block to *GFP* RT products as compared to vector control cells.

The human TRIM5α amino acid sequence comprising the RING and B-box domains was compared to the heterologous TRIM34 sequence (FIG 78A). The alignment shows significant differences in the C-termini of the TRIM34 and TRIM5α RING domains, which might impact on MLV-N restriction. The structures of the TRIM5α and TRIM34 RING domains have been solved and show significant differences (FIG 78B and C), suggesting also differences in the function of both domains.

The data suggest that maximal restriction of MLV-N by human TRIM5α is less dependent on the proteasomal turnover of the protein. The stronger block of T34R5 to MLV-N infection, as compared to human TRIM5α, raised the question whether this increase could be translated to a human TRIM5α protein that was modified in the B30.2 domain to gain specificity for HIV-1 restriction. Mutation of human TRIM5α amino

acid residue R332 to the residue found in rhesus macaque TRIM5α (P332), has been shown to confer the ability to human TRIM5α to restrict HIV-1, although this block is weaker as compared to wild type rhesus TRIM5α [309, 349]. Introducing R332P in T34R5 (FIG 79A) did not result in a significant increase in restriction of HIV-1 or FIV as compared to human TRIM5α R332P (FIG 79C). Comparison of the protein levels in transduced CRFK cell bulks showed that both T34R5 R332P and human TRIM5α R332P were expressed to similar amounts (FIG 79B). Interestingly, mutation of R332P in T34R5 led to a 10-fold decrease in MLV-N restriction (FIG 79C). This observation is in agreement with other work showing that rhesus TRIM5α restricts MLV-N less efficiently compared to human TRIM5α, due to differences in the V1 and V3 regions of the B30.2 domain [306]. The data suggest that MLV-N restriction by human TRIM5α can be manipulated by modification of the RING domain, whereas these modifications do not impact on the restriction of other viruses.

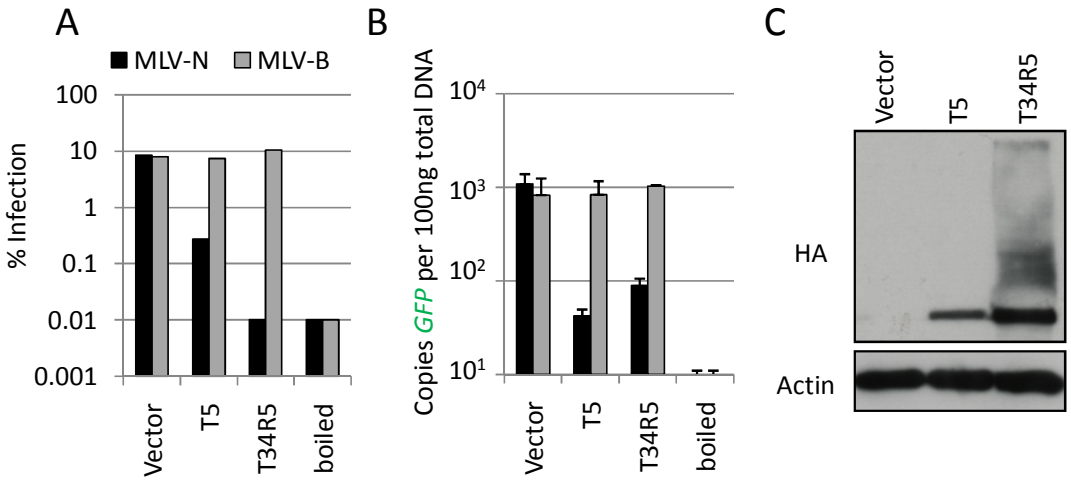

FIG 77 Analysis of the antiviral activity of T34R5

A) CRFK single cell clones transduced with empty EXN vector or vector expressing human TRIM5α (T5) or human TRIM5α with the RING domain substituted to the one from human TRIM34 (T34R5) were infected in triplicate with MLV-N or MLV-B GFP viral vectors and infected cells were enumerated 48 h post infection by FACS. B) Total DNA of two in parallel infected samples was isolated 6 h after infection and GFP copy numbers were measured by Taqman qPCR. As a control, cells inoculated with boiled viral supernatant were analysed. Mean copy numbers per 100 ng total DNA of two in parallel infected samples are shown and error bars are standard deviations of the means. Data are representative of two independent experiments. C) Western blotting to detect HA tagged proteins. Beta actin was used as a loading control.

A

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HuTRIM5   MASGILVNVKEEVTCPICLELLTQPLSLDCGHSFCQACLTANHKKSMLDKG-ESSCPVCR
HuTRIM34  MASKILLNVQEEVTCPICLELLTEPLSLDCGHSLCRACITVSNKEAVTSMGGKSSCPVCG
          *** **:**:*************:*********:*:**:*..:*::: . * :******

HuTRIM5   ISYQPENIRPNRHVANIVEKLREVKLSPE-GQKVDHCARHGEKLLLFCQEDGKVICWLCE
HuTRIM34  ISYSFEHLQANQHLANIVERLKEVKLSPDNGKKRDLCDHHGEKLLLFCKEDRKVICWLCE
          ***. *::::.*:*:*****:*:******: *:* * * :*********:** ********

HuTRIM5   RSQEHRGHHTFL
HuTRIM34  RSQEHRGHHTVL
          **********.*
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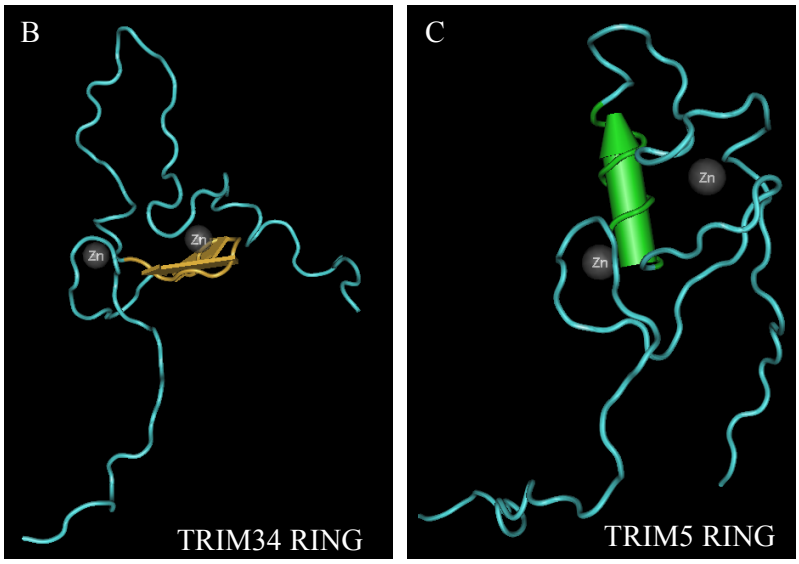

FIG 78 Comparison of the human TRIM34 and TRIM5 RING domains

A) Amino acid alignment of the human TRIM5 and TRIM34 RING and B-box regions. Arrow indicates points of fusion in the tested chimaeric protein. The RING domains are highlighted in yellow and the B-box domains are highlighted in red. The structures of the TRIM34 (C) and TRIM5 (D) RING domains were derived from PDB files 2EGP and 2ECV, respectively, and analysed using the program Cn3D4.1.

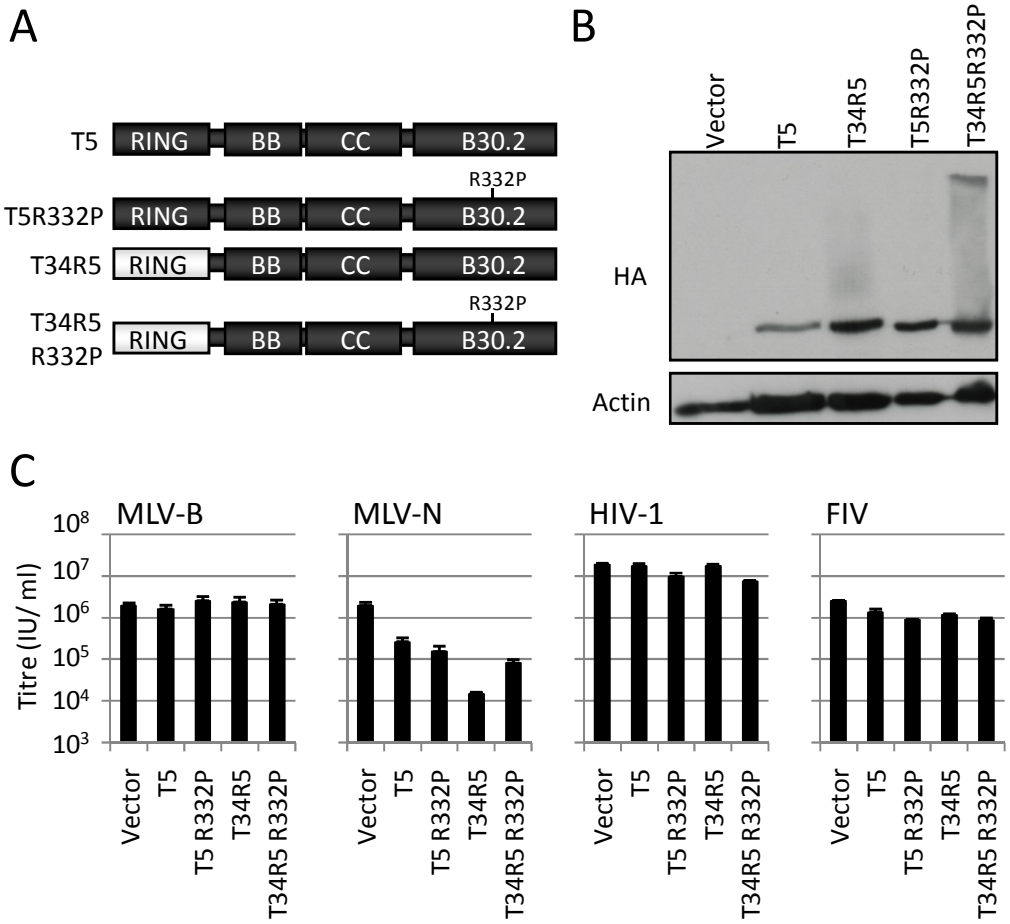

FIG 79 Analysis of T34R5 restriction of HIV-1 in the presence of R332P

A) Schematic representation of the proteins that were expressed in CRFK cells. B) Transduced CRFK cell populations were subjected to western blotting to detect HA tagged proteins. The blot was stripped and re-probed for beta actin as a loading control. C) Vector transduced control cells or cells expressing the different proteins were infected with the indicated GFP vectors. Infectious titres were calculated from three different viral doses and error bars represent standard deviations of the titre means. The data are representative of two independent experiments.

5.2.8. Deletion of the rhTRIM5α RING domain abolishes the block to RT but not to infection

Human TRIM5α restriction depends on the presence of the RING domain. In contrast, it has been reported that RING domain deleted rhesus TRIM5α is still able to restrict HIV-1 infection, however with a diminished block [317, 319, 529]. Maximal restriction of HIV-1 by rhesus TRIM5α therefore depends on the presence of the RING domain. I showed that fusion of the rhesus TRIM5α RING and B-box domains but not fusion of the B-box domain to Fv1Cyp enabled it to block reverse transcription (see 5.2.1.).

In order to better understand the function of the RING domain in rhesus TRIM5α in blocking reverse transcription a RING deletion mutant lacking the first 59 amino acids was generated and expressed in CRFK cells. Drug selected cell populations were then infected with a serial dilution of MLV-B, HIV-1, HIV-2 and FIV GFP vectors and infected cells were enumerated by FACS (FIG 80). In agreement with previous work, deletion of the RING domain (rhΔ59) diminished restriction, although it did not completely abrogate the ability of the protein to block infectivities of HIV-1 or FIV (FIG 80). Interestingly, the block to HIV-2 infection appeared to be slightly more affected by the deletion of the RING domain as compared to HIV-1 or FIV (FIG 80). This result underlines the observation that HIV-2 restriction by rhesus TRIMCyp strongly depends on the presence of the RING domain (FIG 75). SIVmac, MLV-N or MLV-B were neither restricted by wild type rhesus TRIM5α nor the RING deleted mutant (FIG 80). Furthermore, deletion of the first 94 or 129 amino acids in rhesus TRIM5α led to a complete loss of HIV-1 restriction (data not shown).

To analyse the RING deleted rhesus TRIM5α mutant more carefully, CRFK single cell clones expressing rhΔ59 were grown. Five independent single cell clones were then infected in triplicate with HIV-1 or MLV-B GFP vectors. Cell clones rhΔ59c2, c8, c7 and c10 restricted HIV-1 GFP vector by 4-fold, 4-fold, 15-fold and 8-fold, respectively, whereas c1 showed no restriction of HIV-1 (FIG 81A). As a positive control a single cell clone expressing wild type rhesus TRIM5α was infected and showed ~30-fold restriction to HIV-1 infection. MLV-B was not restricted by any of the clones tested (FIG 81B). Taqman quantitative PCR was performed to determine *GFP* copy numbers during restricted infection, as before (FIG 81C and D). The data show that wild type rhesus TRIM5α blocked reverse transcription by ~10-fold (FIG 81C). In contrast, none of three independent cell clones that restricted HIV-1 infection (c2, c7, c10) showed a reduction in the level of *GFP* RT product as compared to infected empty vector control cells (FIG 81C). Reverse transcription of MLV-B was not blocked by any of the cell clones analysed (FIG 81D). Analysis of the expression levels of the deletion mutant in the different single cell clones by western blotting demonstrated that high levels of rhΔ59 were present in the cell clones c2, c7 and c10 that restricted infection (FIG 81E).

These data suggest that as for TRIMCyp, the TRIM5α RING domain mediates the block to reverse transcription. However, for TRIM5α restriction this RING mediated block to RT is crucial for maximal restriction of infection, whereas in TRIMCyp restriction this activity is less important (at least for some viruses).

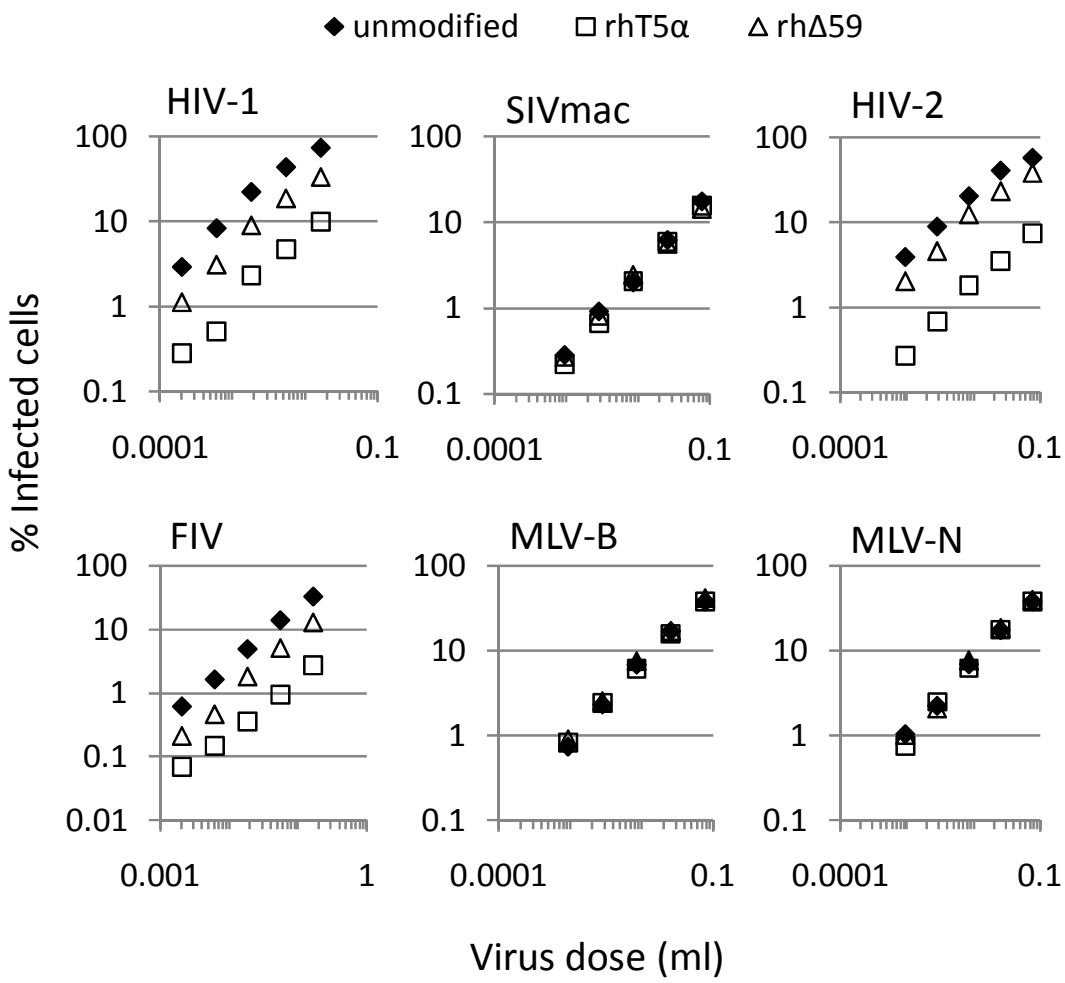

FIG 80 Analysis of a rhesus TRIM5α RING deletion mutant

CRFK cells were transduced with EXN vectors encoding for wild type rhesus macaque TRIM5α or RING deletion mutant rhΔ59 or left untransduced and infected with a serial dilution of indicated GFP viral vectors. Percent GFP positive cells were measured by FACS 48 h post infection and are plotted against the virus dose. The data are representative of two independent experiments.

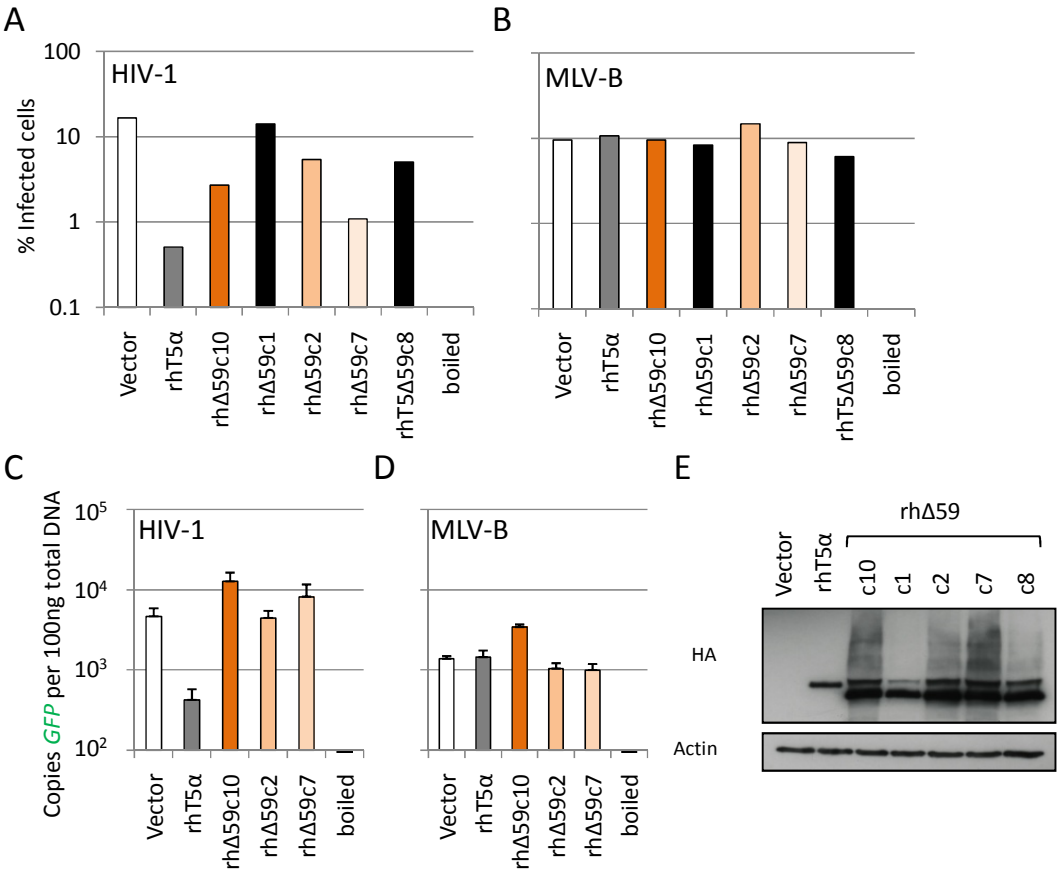

FIG 81 RING deletion mutants of rhesus TRIM5α have lost the block to RT

CRFK single cell clones expressing wild type rhesus TRIM5α or rhΔ59 were infected in triplicate with HIV-1 (A) or MLV-B (B) GFP vectors. Infected cells were enumerated by FACS 48 h post infection (A and B). Two in parallel infected samples were used to determine *GFP* RT copies for the indicated single cell clones by Taqman qPCR (C and D). Boiled supernatants were used as a control for plasmid contamination. E) Western blotting was performed to determine expression levels of the HA tagged proteins in the different single cell clones. Beta actin was used as a loading control. The data are representative of two independent experiments.

5.2.9. RhTRIM5 with a deleted RING domain is inefficiently turned over by the proteasome

One possibility of how the RING domain mediates the block to RT is that it self-ubiquitinates, causing the recruitment of the proteasome and proteasomal degradation of the TRIM5α-viral complex. Inhibiting translation in rhesus TRIM5α expressing cells with cycloheximide and measuring TRIM5α turnover in the presence or absence of proteasome inhibition suggested that TRIM5α is rapidly turned over by the proteasome with a half life of ~60 minutes [518]. Experiments described above demonstrated that deletion of the RING domain in TRIM5α/ TRIMCyp had a similar effect to MG132

treatment of cells expressing wild type TRIM5α/ TRIMCyp in that it rescued reverse transcription [315, 316]. It is therefore possible that the RING domain mediates the block to reverse transcription by self-ubiquitination resulting in rapid turnover by recruitment of the proteasome. To test this hypothesis, CRFK cells expressing wild type rhesus TRIM5α or RING domain deleted protein were treated in a time course with 8 µg/µl MG132 and cell lysates were subjected to western blotting to detect HA tagged protein accumulation.

Inhibition of the proteasome with MG132 for six hours caused the accumulation of modified forms of wild type rhesus TRIM5α, most likely mono- and polyubiquitinated molecules (FIG 82). Interestingly, MG132 treatment of cells expressing rhΔ59 did not result in an increase in the intensity or number of modified forms of the protein, supporting the notion that rhΔ59 is insensitive to proteasome inhibition (FIG 82). Usually, multiple modified forms of rhΔ59, with the most prominent form corresponding to the size of a mono-ubiquitinated protein, were already present in the absence of proteasome inhibition, suggesting that the protein was more stable than the wild type protein. This is in agreement with previous work showing that RING deleted rhesus TRIM5α was less efficiently turned over by the proteasome and has a longer half life [518].

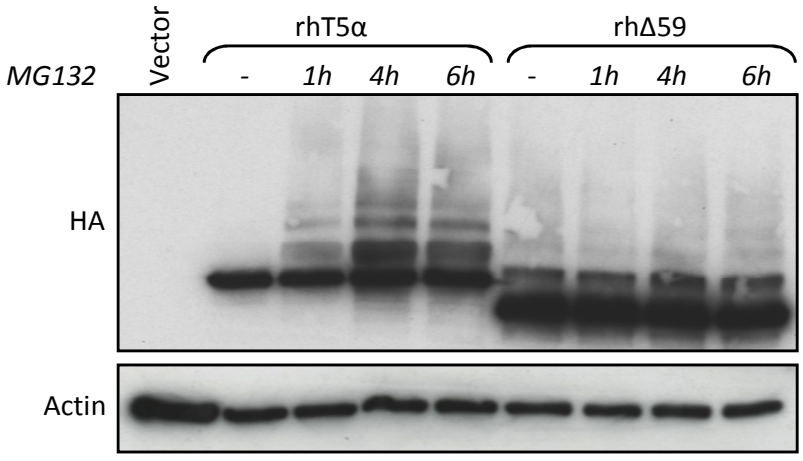

FIG 82 Sensitivity of rhesus TRIM5α and rhΔ59 to proteasome inhibition by MG132 treatment

CRFK cells transduced with empty EXN vector (Vector), rhesus TRIM5α (rhT5α) or RING deleted rhesus TRIM5α (rhΔ59) were treated over a time course of 6 hours with MG132 and western blotting against the N-terminal HA tag was performed on whole cell lysates extracted after indicated time points of MG132 treatment. As a loading control the blot was stripped and re-probed with an antibody against beta-actin. A representative blot of two independent experiments is shown.

5.2.10. RhTRIM5 with a deleted RING domain can still be ubiquitinated

The data shown above suggest that in the absence of the RING domain rhesus TRIM5α is inefficiently turned over by the proteasome leading to a rescue of viral RT products. It is believed that self-ubiquitination of wild type rhesus TRIM5α causes its rapid turnover and degradation by the proteasome resulting in a very short half life of ~60 min [518]. In contrast, RING deleted rhesus TRIM5α has a longer half life [518]. To analyse whether ubiquitination of RING deleted rhesus TRIM5α (rhΔ59) was still possible, an *in vitro* ubiquitination assay was performed (see 2.2.19). 6HIS-ubiquitinated forms of wild type rhesus TRIM5α could be detected after Ni-NTA affinity purification of cell lysates (FIG 83 lane 4). High levels of ubiquitinated rhΔ59 were detected, suggesting that rhΔ59 was still able to serve as a target for ubiquitination (FIG 83 lane 6). Interestingly, a band corresponding to the size of mono-ubiquitinated rhΔ59 was the most prominent. This suggests that the first step during proteasomal TRIM5α turnover may be a RING independent mono-ubiquitination by a yet unknown ubiquitin-ligase. The function of the RING domain might be to transfer the ubiquitin to form poly-ubiquitin chains on the protein. Disruption of the RING function by deletion might therefore lead to an increase of mono-ubiquitinated forms. Both wild type rhesus TRIM5α, as well as rhΔ59 weakly associated with the Ni-NTA resin in the absence of HIS-Ubi, suggesting non-specific binding to the Ni-NTA resin.

The inhibition of the proteasome has been described to increase intracellular TRIM5α aggregation in cytoplasmic bodies or aggresomes [518]. These intracellular structures are very dynamic and cytoplasmic TRIM5α constantly exchanges with TRIM5α in the cytoplasmic bodies [359]. Treatment of cells exogenously expressing tagged TRIM5α proteins of diverse species with geldanamycin, a drug that blocks Hsp90 and disrupts cytoplasmic body formation, showed that cytoplasmic body formation is not required for restriction [357, 522]. It is possible that cytoplasmic bodies represent protein accumulations of excess TRIM5α in the cytoplasm, which is not turned over by the proteasome. The observed ubiquitination of rhΔ59 might then be mediated by other factors present in the cytoplasmic bodies. This hypothesis would suggest that rhΔ59 and TRIM5α expressing cells may differ in their cytoplasmic bodies. To test this, CRFK cells expressing wild type rhesus TRIM5α or rhΔ59 were treated for six hours with 8 µg/µl MG132 or left untreated and used in immunofluorescence staining to detect HA tagged proteins. Empty vector transduced cells were used as a control (FIG 84Ai and Aii). In the absence of proteasome inhibition, wild type rhesus TRIM5α showed a

diffuse cytoplasmic distribution with occasional small cytoplasmic bodies (FIG 84B). After proteasome inhibition by MG132 treatment, the majority of cells showed cytoplasmic bodies increased in both number and size (FIG 84C). In contrast, CRFK cells expressing rhΔ59 showed high numbers of large cytoplasmic bodies even in the absence of MG132 treatment (FIG 84D). Treatment of these cells with MG132 had no effect on the cytoplasmic bodies. The data suggest that rhΔ59 is insensitive to proteasomal degradation causing the accumulation of protein in form of cytoplasmic bodies (FIG 84E). This observation supports the notion that the RING domain mediates effective turnover by the proteasome.

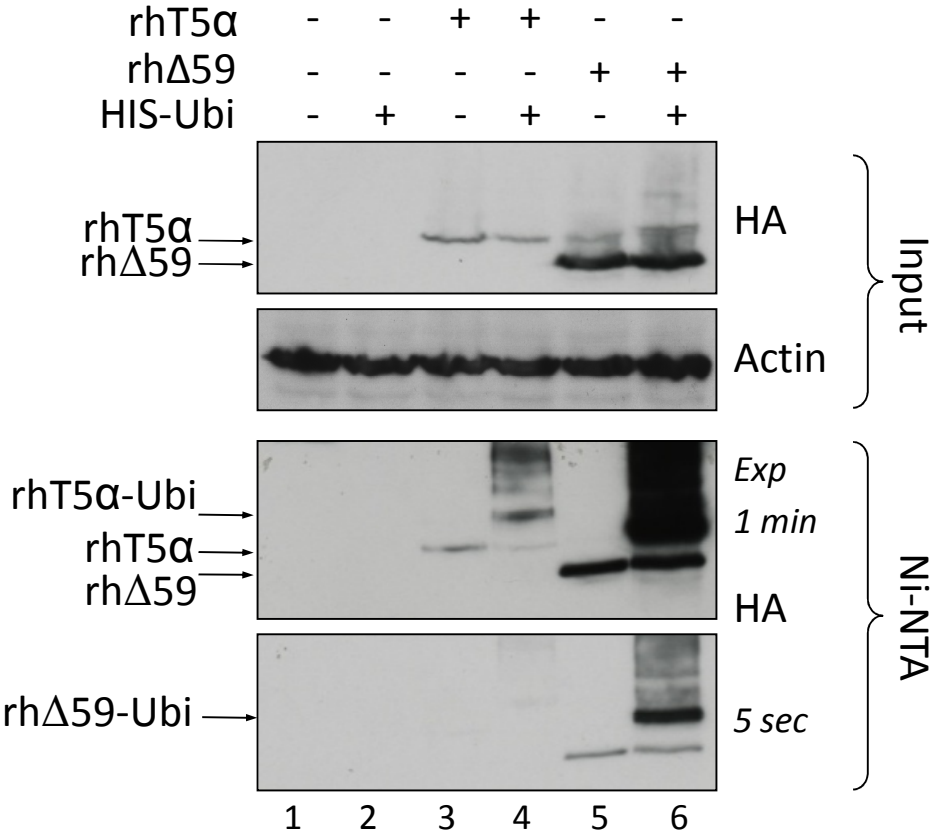

FIG 83 Rhesus TRIM5α with a deleted RING domain is still ubiquitinated

Wild type rhesus TRIM5α (rhT5α) or RING deleted protein (rhΔ59) was co-transfected with 6His-tagged ubiquitin in 293T cells and ubiquitinated proteins were purified by Ni-NTA affinity purification. Input cell lysates and Ni-NTA purified proteins were run on 10% SDS-PAGE gels. HA tagged proteins were detected by western blotting. As a loading control for the input, blots were stripped and re-probed for beta actin. Ni-NTA purified protein blots were exposed for 5 sec or 1 min. The data are representative of two independent experiments.

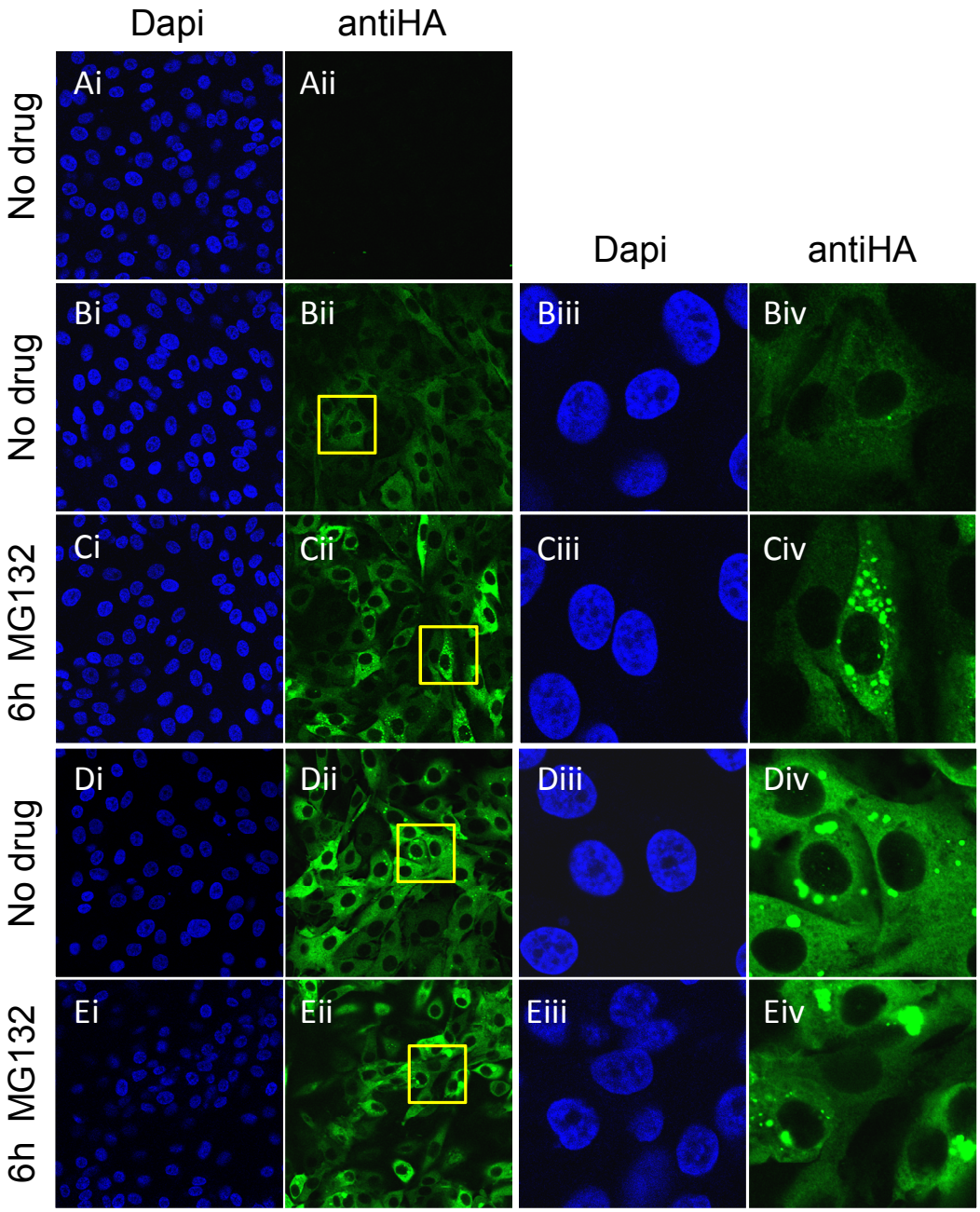

FIG 84 Immunofluorescence analysis of CRFK cells expressing rhT5α or rhΔ59

CRFK transduced with empty EXN vector (A), or EXN encoding wild type rhesus TRIM5α (rhT5α) (B, C), or rhesus TRIM5α with deleted RING domain (rhΔ59) (D, E) were treated for 6 h with 8 µg/ µl MG132 (C, E) or left untreated (A, B, D) and stained with an anti HA antibody after PFA fixation. Pictures were derived using 63× objective (i, ii) or magnified 4 times (iii, iv). Dapi staining (i, iii) shows nuclei, and green fluorescence (ii, iv) shows HA-tagged proteins. The data are representative of two independent experiments.

5.3. Discussion

The aim of this part of the thesis was to determine the function of the RING and B-box domains in TRIM5α and TRIMCyp restriction. To address this, fusion proteins consisting of Fv1Cyp and either the B-box or the RING and B-box domains were generated and characterised. In addition, deletion mutants of different TRIM5α and TRIMCyp proteins were characterised. Furthermore, the RING and B-box domains of human TRIM5α were replaced by heterologous TRIM34 domains and the chimaeric proteins were analysed.

The data presented show that fusion of the RING and B-box domain of rhesus TRIM5 onto the artificial restriction factor Fv1Cyp enables it to block reverse transcription of HIV-1. Interestingly, fusion of the B-box to Fv1Cyp caused a slight increase in restriction. The B-box domain of rhesus TRIM5α has been suggested to mediate higher-order self-association of TRIM5α dimers [536], a function that might also occur for BFv1Cyp and may explain the increase in restriction. However, the block exerted by BFv1Cyp still occurred after the synthesis of reverse transcription products. Interestingly, BFv1Cyp, in contrast to Fv1Cyp, inhibited formation of 2-LTR circles, a possible consequence of an increased higher-order multimerisation mediated by the B-box domain [536], which might decrease the ability of the virus-restriction factor complex to enter the nucleus. Fusion of the RING and B-box domains to Fv1Cyp resulted in a proteasome sensitive block to reverse transcription, suggesting that the RING domain mediates proteasomal degradation by ubiquitination possibly of B-box lysine residues.

Owl monkey TRIMCyp with a deleted RING domain efficiently blocked infection by HIV-1 to a similar degree as wild type omTCyp, a result which is in agreement with other work [319]. Moreover, RING deleted owl monkey TRIMCyp has been shown to be less efficiently turned over by the proteasome than wild type TRIMCyp [518]. Here I show that the block to HIV-1 reverse transcription is significantly reduced in the absence of the RING domain, suggesting that the TRIMCyp RING domain mediates self-ubiquitination leading to degradation by the proteasome. The reason why the RING deleted TRIMCyp still partially restricted reverse transcription might be explained by other TRIM proteins that may be able to cross-ubiquitinate the RING deletion mutant. TRIM21 has been suggested to cross-ubiquitinate TRIM5 and might be a candidate [326]. The reason why BFv1Cyp, unlike RING deleted TRIMCyp, showed no block to

reverse transcription might be explained by the absence of endogenous interaction partners that were able to cross-ubiquitinate BFv1Cyp. The evidence of an involvement of both the RING and the B-box domains in the block to RT are further supported by the observation that a RING and B-box deleted TRIMCyp was significantly impaired in blocking infection, which is in agreement with published work [319]. Importantly, the block to RT was completely abrogated, suggesting an important role for the B-box domain. Furthermore, when owl monkey TRIMCyp mutants deleted in the RING domain or in the RING and the B-box domains were expressed in owl monkey kidney (OMK) cells, they only slightly rescued infectivity of HIV-1. Despite the inability to rescue HIV-1 infection in OMK cells both proteins strongly inhibited the block to reverse transcription mediated by endogenous TRIMCyp. This suggests that the block to reverse transcription depends on two functional RING domains in the TRIMCyp dimer.

Similar results as seen for owl monkey TRIMCyp were obtained for the second naturally occurring TRIMCyp protein from macaques. Unlike owl monkey TRIMCyp, rhesus TRIMCyp efficiently restricts HIV-2 but not HIV-1, in addition to FIV [186]. Deletion of the RING domain had no effect on restriction of FIV infectivity however it partially relieved the block to reverse transcription, as seen for HIV-1 restriction by an owl monkey TRIMCyp RING deletion mutant. Additional deletion of the B-box domain significantly reduced the block to FIV infection and abolished the block to RT. Interestingly, rhesus TRIMCyp restriction of HIV-2 and FIV was differentially dependent on the presence of the RING domain. Deletion of the RING domain almost completely disrupted the block to HIV-2 infection, however only weakly reduced restriction of FIV. Moreover, HIV-1 O-group virus MVP, which was less strongly restricted by rhesus TRIMCyp as compared to HIV-2, was still efficiently blocked in the absence of the RING domain, whereas an HIV-2 mutant that was similarly strongly restricted as MVP was fully infectious in the absence of the TRIMCyp RING domain. These results suggest that some viruses, e.g. HIV-2, are mainly restricted by the RING mediated block, whereas other viruses, e.g. FIV, are mainly restricted by a RING independent block.

The virus specific differences in the requirement for the RING domain in TRIMCyp restriction might be explained by the ability of TRIMCyp to cross-link viral capsid structures. Binding of TRIMCyp to viruses such as HIV-1 and FIV might increase the

stability of their cores due to cross-linking of the CA hexamers. In the presence of the RING domain the cores are directed for proteasomal turnover. In the absence of the RING domain however the viral cores remain cross-linked and uninfectious, but are able to reverse transcribe. In contrast, viruses such as HIV-2 may form different capsid structures and TRIMCyp may be more resistant to cross-linking. Wild type TRIMCyp thus efficiently restricts HIV-2 due to its rapid turnover. Restriction of these viruses might therefore strongly depend on the RING mediated proteasomal turnover. The contribution of the RING mediated proteasomal recruitment in the mechanism of restriction therefore depends on the virus that is restricted.

Deletion of the RING domain of human TRIM5α resulted in the complete loss of MLV-N, EIAV and FIV restriction. This is in agreement with work by Perez-Caballero et al. demonstrating that human TRIM5α with a deleted RING domain was unable to efficiently restrict MLV-N in MDTF cells and moderately rescued infectivity of MLV-N in HeLa cells endogenously expressing human TRIM5α [344]. This suggests that MLV restriction by human TRIM5α is strongly dependent on the presence of the RING domain. Interestingly, MLV-N restriction by human TRIM5α was significantly increased when the human TRIM5 RING domain was replaced with the human TRIM34 RING domain. The human TRIM34 and TRIM5 RING domains differ in their C-termini, which might impact on the differences in MLV-N restriction between wild type human TRIM5α and the chimaeric protein. Moreover, the structures of both domains have been determined and comparison indicates significant differences, which might result in different functions. The results demonstrate that virus specific differential restriction requirements for RING domain function exist for TRIM5α. Although the proteasomal turnover was not measured for the TRIM34-TRIM5 chimearic proteins, previous work suggests that wild type TRIM34 has a significantly longer half life than TRIM5α, possibly a consequence of reduced RING mediated proteasomal turnover of the protein [519]. Other work demonstrated that a chimaeric protein consisting of the RING and B-box domain of human TRIM34 replacing the heterologous domains of rhesus TRIM5α still restricted HIV-1 to rhesus TRIM5α wild type levels [320]. In addition, the same study showed that the rhesus TRIM5α RING domain can be replaced by the heterologous domain of TRIM21, suggesting similar functions between RING domains in the TRIM family [320]. Introducing the mutation R332P into human TRIM5α has been shown to increase restriction of HIV-1 by a few

folds [349]. As shown here, the increase in MLV-N restriction observed for human TRIM5α, in which the RING domain was replaced by the TRIM34 RING domain could not be transferred to HIV-1 when the mutation R332P was introduced. This might be due to a weaker binding of HIV-1 to the human TRIM5α B30.2 domain even in the presence of R332P. The data suggest that MLV-N restriction by human TRIM5α depends on the presence of the RING domain, however might be less dependent on its function. In agreement with that hypothesis, MLV-N reverse transcription was not more strongly inhibited in T34R5 expressing cells as compared to wild type human TRIM5α expressing cells, whereas infectivity was significantly reduced.

Unlike human TRIM5α restriction of MLV-N, HIV-1 restriction by rhesus TRIM5α was not abolished when the RING domain was deleted. In contrast to published work however, deletion of the RING domain resulted in a significant loss of HIV-1 restriction and the block was weaker as compared to wild type rhesus TRIM5α [317, 529]. Previous work showed also that co-expression of wild type rhesus TRIM5α and RING deleted TRIM5α did not rescue infectivity of HIV-1, although both proteins associated efficiently with each other, suggesting that the RING domain is dispensable for restriction of HIV-1 [317]. This observation is similar to results obtained with RING deleted TRIMCyp mutants. High expression of the rhesus TRIM5α RING deletion mutant (rhΔ59) resulted in a moderate block to HIV-1 infection. Importantly, the block to reverse transcription was completely lost. This suggests that, like in TRIMCyp restriction, the block to reverse transcription by TRIM5α is mediated via RING domain function. However, in contrast to restriction by TRIMCyp, maximal restriction of HIV-1 by rhesus TRIM5α was dependent on the presence of the RING domain. This observation might be explained by a higher binding affinity of the CypA domains in a TRIMCyp dimer compared to the B30.2 domains present in a TRIM5α dimer.
Deletion or replacement of the TRIM5α RING domain with heterologous RING domains of other TRIM proteins has been shown to decrease proteasomal turnover leading to a longer half life of the protein [320, 343, 518]. In addition, rhesus TRIM5α with deleted RING and B-box domains has been shown to be less rapidly turned over than a rhesus TRIM5α protein only lacking the RING domain, suggesting that both, an intact RING domain and an intact B-box domain are important for the rapid turn-over by the proteasome [317, 319, 518]. In agreement with these observations, deletion of the N-terminal 94 or 129 amino acids completely abolished the ability of rhesus TRIM5α to

restrict HIV-1. Deletion of the rhesus TRIM5α B-box domain or disruption of zinc coordination by introducing mutations C97A and H100A has been shown to abolish restriction of HIV-1, indicating the importance of the B-box domain in restriction [317, 529]. Interestingly, identical cysteine mutations in an artificial fusion protein of the rhesus RBCC motif with owl monkey CypA did not significantly alter restriction ability against HIV-1 suggesting that the requirement for a functional B-box may depend on the carboxy-terminal virus binding domain [529]. Supporting this idea, one study suggested a functional interplay between the B-box domain with the B30.2 domain, implying that the B-box domain could be involved in regulation of the binding to viral capsids [321]. Recent work proposes that the B-box domain may be involved in co-operative binding and higher-order multimerisation of TRIM5α homodimers [536]. Following this notion the B-box domain might be important for the successive accumulation of TRIM5α homodimers around HIV-1 viral complexes after infection [318].

When wild type rhesus TRIM5α expressing CRFK cells were treated with the proteasome inhibitor MG132 over a time course of six hours, the accumulation of higher molecular weight modified forms of the protein was detected. In agreement with previous studies, the amount of unmodified rhTRIM5α protein did not change during MG132 treatment, however higher molecular weight bands could be detected [315, 518]. In contrast, the RING deleted mutant protein rhΔ59 was insensitive to proteasome inhibition, indicating that the RING domain is important for the proteasomal turnover of the protein. Moreover, NiNTA purification of HIS-Ubi tagged proteins suggested that RING deleted rhesus TRIM5α is still a target for mono- and poly-ubiquitination, possibly mediated by other TRIM proteins. However, since wild type as well as the RING deleted rhesus TRIM5α both bound NiNTA in the absence of HIS-Ubi the possibility that the higher molecular bands represent modifications other than ubiquitin cannot be excluded at the moment. The majority of rhΔ59 was located in large aggregates within cells, which might include other TRIM proteins like TRIM22, TRIM34, TRIM4, TRIM6 and TRIM27 [519]. Why cross-ubiquitination did not result in efficient proteasomal degradation of rhΔ59 is unclear. It is possible that the transfer of an ubiquitin monomer to TRIM5α by an E2 ubiquitin ligase might trigger the RING dependent poly-ubiquitination of TRIM5α causing the recruitment of the proteasome. In the absence of the RING domain, the mono-ubiquitinated protein was the most

prominent modified form and poly-ubiquitination by other TRIM proteins may not be sufficient for rapid proteasomal degradation.

In conclusion, both the RING and the B-box domains are important for the proteasome dependent block to reverse transcription exerted by TRIM5α/ TRIMCyp. The RING domain likely mediates self-ubiquitination, maybe at B-box lysine residues, leading to proteasomal degradation of the restriction factor-capsid complex. Deletion of the RING domain recapitulates the rescue of reverse transcription of restricted virus by a drug induced block of the proteasome, suggesting that the RING domain mediates the recruitment of the proteasome. In TRIM5α this block represents the major barrier to HIV-1 whereas in TRIMCyp this block is differentially important, depending on the restricted virus. The restriction of some viruses strongly depends on the presence of the RING domain, whereas restriction of other viruses is less dependent on the RING domain. Importantly this is independent of the strength of the block. Deletion of the RING and the B-box domains completely abrogates restriction by TRIM5α and significantly impairs restriction by TRIMCyp indicating that both domains co-operate to exert TRIM5α/ TRIMCyp antiviral activities.

6. Future work

Future work will focus on the identification of the full-length hare TRIM5 cDNA and its characterisation and comparison with rabbit TRIM5, which might provide insights into the antiviral mechanism and specificity of restriction. Furthermore, it would be interesting to test whether a viral vector expressing a reconstructed RELIK capsid is sensitive to restriction by rabbit or hare TRIM5. In addition, the effect of cyclosporine on HIV-1, HIV-2 and FIV infectivities in rabbit and hare cells needs further investigation. As demonstrated here the rescue of infectivities of HIV-2 and FIV by addition of cyclosporine in rabbit cells may be independent of rabbit TRIM5 activity, and further characterisation may reveal insights into functions of other cyclophilins on lentiviral infection.

TRIMCyp fusion proteins have been useful in identifying interaction partners with HIV-1 capsid, e.g. the Nup358 Cyp domain. This could be used as a tool and cDNA libraries could be cloned into the Cyp position with a view to identify cellular proteins that bind capsid which might reveal novel factors involved in early processes of HIV-1 infection, although this may be technically challenging. Furthermore, Nup358 has been demonstrated to be involved in nuclear import of HIV-1. I showed here that the Cyp domain of Nup358 can recruit lentiviral capsids when expressed in a TRIMCyp context. It will be investigated by crystallography whether monomeric Nup358 Cyp also binds HIV-1 capsids. In addition, siRNA mediated reduction of Nup358 and infection with different HIV-1 CA mutants will reveal whether CA binding to Nup358 Cyp is important for nuclear import of HIV-1. A major project will be to investigate whether CA is present in HIV-1 PICs and its role in nuclear import.

Future work on the mechanism of TRIM5α restriction will try to understand the role of the ubiquitin-/ proteasome pathway. Immunoprecipitation of HA tagged wild type and RING deleted TRIM5α with an anti-HA antibody and subsequent detection of ubiquitin using and anti-ubiquitin antibody will clarify whether both proteins get ubiquitinated as suggested by the NiNTA purification experiments. Furthermore, SiRNA screens will be performed to identify the E2-ubiquitin ligase(s) that target TRIM5α. In addition, purification of large amounts of 6HIS-ubiquitinated TRIM5α and subsequent mass spectrometry analysis will identify the TRIM5α residues that are targeted for

ubiquitination. This analysis will answer whether ubiquitin is linked to K63 or K48, maybe revealing differences in the restriction mechanism. Subsequent analyses by mutating the identified lysine residues and measuring the block to reverse transcription and infection will clarify the role of the ubiquitin-proteasome pathway. Although the B-box domain has been suggested to mediate higher-order homomultimerisation of TRIM5α dimers it is not clear whether it may also support heteromultimerisation, e.g. with TRIM21, which might impact on the antiviral activity of TRIM5α. A crystal structure of TRIM5α in complex with HIV-1 hexameric CA will likely reveal insights into inter- and intramolecular interactions.

It may also be interesting to investigate more precisely the role of cytoplasmic bodies in the restriction mechanism. Although pre-existing cytoplasmic bodies are not required for restriction, TRIM5α/TRIMCyp aggregates around viral complexes after infection, suggesting that cytoplasmic bodies are involved in the restriction mechanism. Purification of cytoplasmic bodies and identification of other cellular factors that are present could be important in understanding the role of these aggregates in TRIM5α/TRIMCyp mediated viral restriction as well as a possible cellular role of TRIM5α in the absence of virus infection.

7. Figures

8. Tables

9. References

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